

Cytokine mediated CD4⁺ T-cell dysregulation in early rheumatoid arthritis

Thesis for the degree of Doctor of Philosophy

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Abstract

Background

A 12-gene signature in CD4⁺ T-cells had discriminatory utility for early rheumatoid arthritis (RA) patients compared with disease controls. This signature is enriched for STAT-3 target genes whose expression correlates with paired circulating IL-6. I hypothesise that pre-exposure of CD4⁺ T-cells to IL-6 mediates STAT-3 activation and aberrant effector function following T-cell receptor (TCR) stimulation, providing a mechanism of antigen non-specific immune dysfunction in early RA.

Methods

A model for cytokine pre-exposure was developed, in which naïve (CD45RA⁺) and antigen experienced (CD45RA⁻) CD4⁺ T-cells from healthy human donors were cultured with IL-6 and equimolar soluble IL-6R for 3 days, before being washed and stimulated with anti-CD3 and anti-CD28 for 6 days. RNA was extracted at multiple experimental time-points and global gene expression profiling undertaken. Phenotype and proliferation were assessed by flow cytometry, measuring cell surface markers and proliferation dyes. Whether the observed consequences of IL-6 pre-exposure reflected transcriptional and phenotypic characteristics of CD4⁺ T-cells isolated from patients with early RA was explored.

Results

The effects of IL-6 pre-exposure were seen most prominently in naïve CD4⁺ T-cells, potentially related to IL-6 receptor expression. Pre-exposure of healthy control naïve CD4⁺ T-cells to physiological levels of IL-6 caused significant STAT-3 target gene induction, mirroring genes previously found to distinguish RA patients from disease controls. Following TCR-stimulation, a distinctive set of genes differentially expressed in IL-6 pre-exposed cells were associated with cell proliferation and survival. This is consistent with altered effector phenotype of IL-6 pre-exposed cells characterised by dose-dependent enhancement in activation and proliferative capacity. CD4⁺ T-cells from early RA patients showed no difference in proliferation compared to healthy controls, although our group has observed that a higher proportion are committed to the cell cycle as measured by ki67 expression.

Conclusion

These findings highlight that cytokine “pre-priming” during the early disease state may have consequences for naïve CD4⁺ T-cell effector function, impacting the transition to disease chronicity in early RA.

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List of Abbreviations

³ H-TdR	Tritiated thymidine
AA	Adjuvant induced arthritis
AIA	Antigen induced arthritis
AICD	Activation induced cell death
Aire	Autoimmune regulator
ACPA	Anti-citrullinated peptide antibody
ADAM	A disintegrin and metalloprotease domain
ANKRD	Ankyrin repeat domain
APC	Antigen presenting cell
APP	Acute phase proteins
APR	Acute phase response
BCL	B-cell lymphoma
BFA	Brefeldin A
BLK	B lymphocyte kinase
BMI	Body mass index
BSA	Bovine serum albumin
CII	Type II collagen
CCL	Chemokine ligand
CCP	Cyclic citrullinated peptide
CCR	Chemokine receptor
CD	Cluster of differentiation
CD	Crohn's disease
cDNA	Complementary DNA
CFSE	Carboxyfluorescein succinimidyl ester
ChIP	Chromatin immunoprecipitation
CIA	Collagen induced arthritis
CIS	Cytokine inducible SH2
CLC	Cardiotrophin-like cytokine
c-maf	Musculoaponeurotic fibrosarcoma oncogene homolog
CNTF	Ciliary neurotropic factor
CPM	Counts per minute
CRP	C-reactive protein

CSA	Clinically suspect arthralgia
CT1	Cardiotrophin-1
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
CXC-(L/R)	C-X-C chemokine (ligand/receptor)
DAMP	Damage associated molecular pattern
DAPI	4', 6-diamidino-2-phenylindole
DAS28	Disease activity score
DC	Dendritic cell
DMARD	Disease modifying anti-rheumatic drug
DMSO	Dimethyl sulfoxide
DNA	Deoxynucleic acid
dNTP	Deoxyribose nucleoside triphosphate
DSS	Dextran sodium sulphate
DTT	Dithiothreitol
EA	Early arthritis
EAE	Experimental autoimmune encephalomyelitis
EAU	Experimental autoimmune uveoretinitis
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immune-sorbent assay
eQTL	Expression quantitative trait loci
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinases
FACS	Fluorescence-activated cell sorting
Fc	Fragment crystallisation
FCS	Fetal calf serum
FEZF2	FEZ family zinc finger 2
FITC	Fluorescein isothiocyanate
FMO	Fluorescence minus one
Foxp3	Forkhead box p3
FSC-A	Forward scatter area
GAB1	Grb2-associated binder 1
GATA-3	GATA-binding protein-3
GC	Germinal centre
G-CSF	Granulocyte-colony stimulating factor

GM-CSF	Granulocyte-macrophage colony-stimulating factor
GP	Glycoprotein
GPI	Glucose-6-phosphate isomerase
GR	Glucocorticoid receptor
GRB	Growth factor bound protein 2
GWAS	Genome wide association study
HLA	Human leukocyte antigen
HBSS	Hanks balanced saline solution
HRP	Horseradish peroxidase
IBD	Inflammatory bowel disease
ICAM	Intracellular adhesion molecule
ICOS	Inducible T-cell co-stimulator
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
ILC	Innate lymphoid cells
IL-6R	Interleukin-6 receptor
IMDM	Iscove's modified dulbecco's medium
JAB	Janus kinase binding protein
JAK	Janus kinase
JIA	Juvenile idiopathic arthritis
JNK	c-Jun N-terminal kinases
KO	Knock out
L	Ligand
LAP	Latency associated peptide
LIF	Leukaemia inhibitory factor
LPS	Lipopolysaccharide
LRS	Leukocyte reduction system
mAb	Monoclonal antibody
MACS	Magnetic activated cell sorting
MAPK	Mitogen activated protein kinase
MCP	Monocyte chemotactic protein
MHC	Major histocompatibility complex
MK2	Mitogen activated protein kinase activated protein kinase 2

MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MS	Multiple sclerosis
MSD	Meso Scale Discovery
mTEC	Medullary thymic epithelial cell
MTX	Methotrexate
MUC	Mucin
NFAT	Nuclear factor of activated T-cells
NF- κ B	Nuclear factor kappa-light-chain-enhancer of activated B-cells
NK	Natural killer
NLS	Nuclear localisation signal
NNT-1	Neurotrophin
NOD	Non-obese diabetic
NPN	Neuropoietin
OA	Osteoarthritis
OPD	o-Phenylenediamine dihydrochloride
OR	Odds ratio
OSM	Oncostatin M
p	phosphorylated
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Programmed cell death protein
PDGF	Platelet derived growth factor
PHA	Phytohaemagglutinin
PI3K	Phosphoinositide-3 kinase
PIAS	Protein inhibitor of activated STAT
PIM	Pro-viral integration site for Moloney murine leukemia virus
PKB	Protein kinase B
PKC	Protein kinase C
PMA	Phorbol 12-myristate 13-acetate
POLR2A	RNA polymerase II subunit A
PTP	Protein tyrosine phosphatase
PTPN	Protein tyrosine phosphatase non-receptor

qPCR	Quantitative real-time PCR
RA	Rheumatoid arthritis
RANKL	Receptor activator of nuclear factor kappa B ligand
RAPS	Rheumatoid associated peptide
RBC	Red blood cells
RF	Rheumatoid factor
RNA	Ribonucleic acid
ROR	RAR related orphan receptor
ROS	Reactive oxygen species
RPMI	Roswell park memorial institute medium
SAA	Serum amyloid A
SBNO2	Strawberry notch homolog 2
SSC-(A/W)	Side scatter (area/width)
SCID	Severe combined immunodeficiency
SE	Shared epitope
SEM	Standard error of the mean
SF	Synovial fluid
SGK	Serum/glucocorticoid regulated kinase
SH2	Src homology region 2
sIL-6R	Soluble IL-6 receptor
SLE	Systemic lupus erythematosus
SLO	Secondary lymphoid organ
SNP	Single nucleotide polymorphism
SOCS	Suppressor of cytokine signalling
SOS	Sons-of-Sevenless
SR	Serum replacement
SS	Sjögren's syndrome
SSI	STAT inducible STAT inhibitors
SSZ	Sulfasalazine
ST	Synovial tissue
STAT	Signal transducer and activator of transcription
T1D	Type 1 diabetes
T2DM	Type 2 diabetes mellitus
TACE	Tumour necrosis factor a converting enzyme

T-bet	T-box transcription factor
T _{CM}	Central memory T-cell
TCR	T-cell receptor
TCZ	Tocilizumab
Teff	Effector T-cell
T _{EM}	Effector memory T-cell
T _{EMRA}	Terminally differentiated T-cell
T _{FH}	T-follicular helper cell
TGF	Transforming growth factor
Th1	T-helper cell 1
Th2	T-helper cell 2
Th9	T-helper cell 9
Th17	T-helper cell 17
Th22	T-helper cell 22
TIMP	Tissue inhibitors of matrix metalloproteinases
TLDA	TaqMan low density array
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFAI3	Tumour necrosis factor- α induced protein 3
TREC	T-cell receptor excision circle
Treg	Regulatory T-cell
UC	Ulcerative colitis
VDJ	Variable Diversity Joining genes
VEGF	Vascular endothelial growth factor
WTCCC	Welcome trust case control consortium
ZAP	Zeta chain associated protein kinase 70

Chapter 1. Introduction

1.1 Rheumatoid Arthritis

Rheumatoid arthritis (RA) is a chronic inflammatory disease in which painful and persistent synovial inflammation in symmetrically distributed small joints typically results in tissue damage and disability. Although sometimes termed an autoimmune disease, no disease specific auto-antigen explains the observed immune dysregulation and the cause of peripheral tolerance breakdown is not yet understood (1).

1.1.1 Epidemiology and Heterogeneity

RA is the most common inflammatory joint disease in humans with a population prevalence of 0.5-1.1% and a mean annual occurrence of 0.02-0.05% (2). In several populations, including the UK, there is an apparent decline in RA prevalence and incidence (3). The peak incidence occurs between the ages of 40 and 50 and occurs more frequently in women compared to men with a sex ratio of between 2:1 and 3:1 (3).

Patients with RA have an increased risk of co-morbidities with cardiovascular disease, myocardial infarctions and bacterial, fungal and viral infection rates disproportionately increased in RA (4). This ultimately influences quality of life and increases mortality. Timely treatment may reduce disease activity as well as incidence of comorbidities. Furthermore, delays in the diagnosis and treatment of RA results in adverse outcomes with studies showing that a high level of joint damage occurs within the first year of disease onset (5). This period is often described as a window of opportunity in which early treatment results in better response (6-8). For this reason, there is continued investigation into the discovery of biomarkers that can be used to predict disease – particularly in the substantial proportion of patients attending an early arthritis clinic with unclassifiable “undifferentiated” inflammatory arthritis.

The clinical presentation of RA is very heterogeneous, posing challenge to clinicians seeking early diagnosis and effective treatment (9). For example, it is becoming increasingly clear that the disease can be categorised into at least 2 subgroups, seropositive or seronegative, depending on the presence or absence of circulating autoantibodies respectively, but even within these sub-populations, a high degree of variability remains (10).

The natural history of seronegative RA progression differs from that of seropositive disease with late and explosive onset being well documented (10). The proportion of RA patients who are seronegative is now estimated to be around 0.22 (11).

It is increasingly being understood that heterogeneity of the disease extends beyond serotype and clinical presentation to include synovial pathology and treatment responsiveness. A lower rate of joint destruction has been documented in seronegative disease with lower levels of lymphocyte infiltration and lower levels of T-cell derived pro-inflammatory cytokines in synovial fluid (SF) (12, 13). Some treatments have demonstrated increased efficacy in seropositive individuals, which may reflect the differences in pathology (14, 15). This suggests a wide range of molecular and cellular mechanisms contribute to pathology, and these are likely to be distinct but slightly overlapping in the RA serotypes underlying the differences seen between the two subgroups.

1.1.2 Genetic Factors Influencing RA

Genetics has long been known to play an important role in the development of RA. Twin studies highlight the role of genetics with concordance among monozygotic twins around 15-30% compared to 3.6% for dizygotic twins (16, 17). The heritability of RA has been estimated to be 60% for seropositive disease but the heritability of seronegative disease is thought to be much less (18, 19).

The region of the genome on chromosome 6 encoding the major histocompatibility complex (MHC), involved in the process of antigen presentation, has been shown to have strong associations with RA (20). This region encodes MHC class I molecules such as human leukocyte antigen (HLA)-A, HLA-B and HLA-C recognised by cluster of differentiation 8 (CD8⁺) T-cells and MHC class II molecules encoding HLA-DR, HLA-DQ, HLA-DP recognised by CD4⁺ T-cells. A strong genetic association can be seen with the HLA-DR alleles with over 80% of RA patients carrying variants of the HLA-DRB1*04 alleles (21). HLA risk alleles across 3 loci encode a common 5-amino acid sequence within the hypervariable region of MHC II, which is the site of the peptide binding groove important for antigen binding and presentation to CD4⁺ T-cells, these explain the majority of the HLA association to RA (22). These sets of alleles are known as the “shared epitope” (SE) and confer susceptibility to RA and increase disease severity with odds ratios (OR) of 4.37 for heterozygotes and 11.79 for homozygotes (23-25). Although many variants of the DRB1 allele confer susceptibility others have also been shown to confer protection against RA, namely HLA-DRB1*13 (26). The SE is particularly associated with seropositive disease, which may be due to an increased ability of citrullinated peptides to bind HLA (27, 28). It is now suggested HLA-DRB1 alleles provide the basis for the development of self-recognising auto-reactive T-cells that respond to anti-cyclic citrullinated peptide (CCP) formation (29). Conversely, in seronegative disease the HLA region confers relatively little risk, although the DRB1*13 allele conferring protection from

seropositive disease has been shown to work in combination with DRB*03 to increase the susceptibility of seronegative disease (30).

The HLA region accounts for only around a third of the genetic contribution to RA meaning there are many more associations in other parts of the genome (31). Genome wide association studies (GWAS) confirm the association of HLA-DRB1 and protein tyrosine phosphatase-22 (PTPN22), which together explain 50% of familial aggregation (32). The association of PTPN22 involves a missense substitution of arginine to tryptophan at residue 620, R620W, which results in enhanced regulation of CD4⁺ lymphocyte T-cell receptor (TCR) signalling resulting in the presence of auto-reactive T-cells in the periphery (33, 34).

Further associations with non-HLA regions of the genome have been discovered including cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and signal transducer and activation of transcription-4 (STAT-4) (35, 36). CTLA-4 is a CD28 homologue found on the surface of T-cells involved in down-regulating T-cell co-stimulation and activation. One of the most studied polymorphisms involves the substitution of threonine to alanine at position 49, T49A, which results in incomplete glycosylation and lower surface levels (37). STAT-4 is a transcription factor activated by interleukin-12 (IL-12) and IL-23 signalling, and a haplotype of this gene has been associated with susceptibility to RA. This molecule can activate interferon- γ (IFN- γ) genes which play a key role in T-cell differentiation (36, 38, 39).

Many of the non-MHC risk alleles identified are associated with seropositive disease, in contrast, for seronegative disease only 15 non-MHC risk alleles have been identified including ankyrin repeat domain 55 (ANKRD55) and B lymphocyte kinase (BLK) with OR of 0.8 and 0.13, resulting in protective effects or increased risk, respectively. This highlights the genetically distinct nature of these diseases (10).

The total number of disease associated SNPs now stands at 101 and a disproportionate number of these are found at loci containing genes whose products are involved in T-cell function (40). Many of these SNPs overlap with CD4⁺ T-cell specific chromatin marks providing evidence of the importance of CD4⁺ T-cells in RA pathogenesis (41). Although risk conferred by these disease associated SNPs alone is small, presence of a number of these loci may confer higher risk. Most likely, the development of RA will come from a combination of both genes and environmental factors.

1.1.3 Environmental Influences

The most prominent environmental influence on the development of RA is smoking (42). The increased amount and duration of smoking has been shown to cause dose-dependent increased

risk of RA (43, 44). The influence of smoking is restricted to seropositive RA and is much greater in males compared to females with smoking increasing male OR from 1.89 to 3.02 (45-47). Smoking causes multiplicative risk in individuals with HLA-DRB1 alleles with a dose effect with the number of risk alleles. In contrast, the consumption of alcohol has been described to have a protective influence on the development of seropositive RA with one study showing the highest level of alcohol consumption resulted in half the risk of disease (48).

An environmental factor heavily linked to the development of seronegative RA is obesity. A body mass index (BMI) of over 30 for the decade prior to RA onset causes triple the risk of developing disease with an OR of 3.45 (49). Many other environmental influences have been described as contributing to the risk of developing RA; however, these have much less supporting evidence. For example, oral contraceptive use, high levels of vitamin D intake and previous blood transfusions have all been shown to both increase and decrease risk of RA (50).

It is now generally believed that genetics pre-dispose towards RA but an environmental factor may play an important role in triggering disease, although the relative importance of these factors is likely to differ between seropositive and seronegative disease. For example, in seropositive disease it is likely that environmental factors influence citrullination and the HLA association confers the risk of mature autoantibody response.

1.1.4 Role of the Immune System in RA

The synovium is the layer of connective tissue that lines the inner surface of diarthrodial joints. The synovium consists of two layers: the underlying tissue, the subintima, and the continuous layer of surface cells, the intima. These synovial lining cells consist of fibroblast-like and macrophage-like synoviocytes, which maintain the composition of the SF filling the joint cavity. This SF provides an important source of nutrition for cartilage by transporting substances such as oxygen, glucose, proteinases and collagenases and provides lubrication to reduce friction between cartilage during movement.

RA is characterised by inflammation of the synovium beginning with hypertrophy of the synovial lining with proliferation of fibroblast-like and macrophage-like synoviocytes (1). Concurrently, angiogenesis and infiltration by inflammatory cells occur. The result of this infiltration is the formation of the pannus, a leading edge of invasive tissue comprising proliferating synovial fibroblasts and inflammatory cells. As a result of pro-inflammatory cytokine release by this tissue, including IL-1, tumour necrosis factor (TNF) and matrix metalloproteinases (MMPs), other proteolytic enzymes are released by chondrocytes causing matrix destruction and osteoclast activation leads to bone degradation, *Figure 1* (51). The

pathogenesis of RA is still not fully understood but cannot solely be attributed to one cell type or pathway. It is likely that the contribution of different immune cell subsets to pathogenesis varies between individuals.

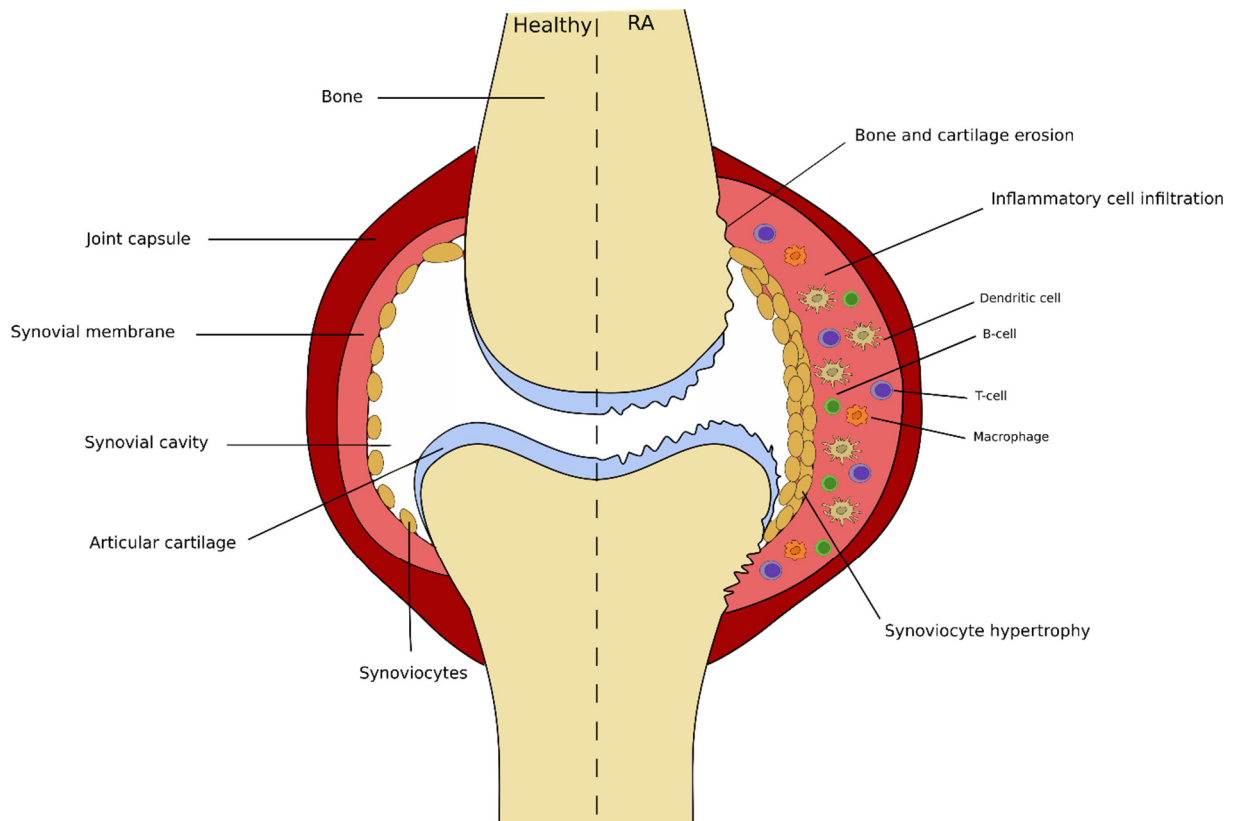


Figure 1. Healthy joint versus RA joint. In rheumatoid arthritis there is hypertrophy of synovial lining, influx of inflammatory cells into the synovial membrane and proinflammatory cytokine release. These factors then lead to the destruction cartilage and bone.

1.1.4.1 Antigen presenting cells

Dendritic cells (DC) are professional antigen presenting cells (APCs) that present antigen via the MHC class II, hence are implicated in RA pathogenesis by the shared epitope association. DC infiltration occurs early in RA pathology with high numbers found in SF and synovial tissue (ST) (52). DCs are also highly abundant in T-cell clusters and their high levels of MHC class II and costimulatory molecules stimulate T-cell responses and perpetuate inflammation (52). Current therapy in RA methotrexate (MTX) has also been shown to alter the number and function of mature DCs (53).

1.1.4.2 B-cells and Autoantibodies

Due to the presence of autoantibodies, B-cells have long been thought to play a role in RA pathogenesis as antibodies against citrullinated proteins have been shown to amplify tissue injury (54). Further evidence for a role for B-cells in RA is the presence of germinal centre

(GC) like structures in the inflamed synovium in around 30-50% of patients (55). B-cells in these GCs act as APCs to CD4⁺ T-cells perpetuating immune responses (56-58). Moreover, the depletion of B-cells has been successful in the treatment of RA, with reduced radiographic progression and improvements in disease activity score 28 (DAS28) scores, with a higher efficacy in seropositive RA (59, 60).

Evidence that activation of the adaptive immune system by “self” antigens drives arthritis in RA is well-established; for example autologous SF induces proliferation of T-cell clones isolated from joints of affected patients (61). Although RA has been termed an autoimmune disease, many studies have failed to link tissue specific antigens, such as type 2 collagen, cartilage protein glycoprotein 39 (gp39) and proteoglycans, to the disease (62-64). Autoantibodies however, have been associated with RA such as anti-citrullinated peptide antibody (ACPA) and rheumatoid factor (RF) (65).

Presence of ACPA are over 95% specific for RA and are found in less than 2% of healthy individuals (66). ACPA recognise post-translationally modified (citrullinated) epitopes such as on fibrogen, fibrinogen, vimentin, histones and collagen. Citrullination occurs naturally in the body as a result of peptidyl arginine deaminase dependent conversion of arginine to citrulline (67). The presence of ACPAs towards these citrulline residues has been shown to predict a worse clinical outcome with promotion of bone erosion by activation of osteoclasts, and in mouse models, these molecules have been shown to induce pain and enhance synovitis (54, 68, 69).

The presence of RF is closely linked to RA with 74% specificity, however, can be found in other autoimmune diseases such as Sjögren’s syndrome (SS), osteoarthritis (OA) and chronic infections, limiting the specificity of this autoantibody, although it is still used to aid diagnosis (70). This autoantibody recognises fragment crystallisation (Fc) segments of immunoglobulin G (IgG) molecules creating crosslinks resulting in the formation of immune complexes which can trigger complement activation (71). This innate response activates inflammation, lysis and immune clearance enhancing antigen presentation so is thought to play a role in disease pathogenesis (72, 73).

The presence of ACPA and RF autoantibodies frequently predates clinical onset of disease, and predicts a more severe form of disease with higher rate of joint destruction (65, 74, 75). Despite the use of ACPA autoantibodies in RA diagnosis the absence of both autoantibody classes, termed seronegative or RF/ACPA-negative RA, accounts for about 20% of the overall disease

spectrum. The absence of autoantibodies contributes to diagnostic delays in this group, which may also be subject to sub-optimal treatment responses (76).

1.1.4.3 T-cells

T-lymphocytes are key mediators of cellular responses and recognise antigen presented on the surface of antigen presenting cells such as DCs by the MHC, via their TCR.

These cells are derived from hematopoietic stem cells in the bone marrow and migrate to the thymus where they are able to mature from double negative cells, which do not express the CD3 T-cell receptor (TCR) or CD4/CD8, into T-cells expressing combinations of CD3, CD4 and CD8. The interactions with MHC molecules helps to shape the TCR repertoire and surface molecule expression by determining whether these cells are single positive cells expressing CD3⁺ and either CD4⁺ or CD8⁺, with CD8⁺ T-cells recognising MHC class I and CD4⁺ T-cells recognising MHC class II (77). The TCR consists of $\alpha\beta$ or $\gamma\delta$ chains bonded with five subunits of CD3. Gene rearrangements between variable, diversity and joining (V-D-J) genes generates a diverse repertoire of TCR unique to individual cells (78).

The microenvironment of the thymus is crucial for the generation of functional T-cells. The expression of self-antigens by medullary thymic epithelial cells (mTEC), regulated by the expression of autoimmune regulator (Aire) and FEZ family zinc finger 2 (FEZF2), promote tissue specific antigen expression to educate T-cells for the elimination of auto-reactive T-cells (79). During maturation in the thymus, T-cells undergo positive and negative selection, in the cortex and medulla respectively. During positive selection, T-cells that fail to recognise self-MHC molecules are deleted. Those cells recognising self-MHC next undergo negative selection, where cells with strong responses to self-antigens are deleted, and those with intermediate TCR affinity are selected (78).

T-cells are strongly implicated in RA pathogenesis with the high abundance of CD4⁺ and CD8⁺ lymphocytes in the synovium of RA patients (12, 80, 81). Due to the focus of this thesis, CD4⁺ T-cells will be dealt with in more detail in *Chapter 1.2*.

CD8⁺ T-cells are also known as cytotoxic T-cells as these cells are responsible for the killing of pathogenic cells via the release of cytotoxins such as perforin and granzymes. CD8⁺ T-cells make up 40% of all T-cells in the SF and play a crucial role in the formation of germinal centres in the synovium (82). The role of CD8⁺ T-cells in the pathogenesis of RA is poorly understood, however, it has been documented that peripheral blood CD8⁺ T-cells from RA patients appear to have an increased proportion of central memory cells and increased numbers of regulatory T-cells which appear to correlate with DAS28 (83, 84).

1.2 CD4⁺ T-cells

Following thymic development, naïve CD4⁺ T-cells enter the circulation and are maintained by low affinity interaction of TCR with self-antigen. These cells can be identified by the expression of CD45RA, a molecule involved in cell signalling, which is alternatively spliced to CD45RO following T-cell activation. Naïve cells migrate towards secondary lymphoid organs (SLOs) such as lymph nodes, spleen and tonsils, due to expression of lymph node homing receptors such as CD62L and C-C chemokine receptor type 7 (CCR7), where APCs can display antigen via the MHC class II to induce an adaptive response. In addition to the TCR-MHC interaction, additional signals are required for T-cell activation including co-stimulation, where CD28 on CD4⁺ T-cells interact with CD80/CD86 on APCs and signals from cytokines in the surrounding environment (85, 86).

Activated cells will then proliferate, differentiate and leave the SLOs as effector cells. T-cells will divide to produce identical progeny, termed clonal expansion (87). The type of effector cell generated depends on the concentration of antigen, number of costimulatory molecules involved, cytokines present during stimulation and the DC subset (88-90).

Different subsets of T-helper cells can be differentiated which provide signals to B-cells for maturation, maintain CD8 responses and regulate innate effector cells. These T-helper subsets, which include but are not limited to Th1, Th2, Th17 and Treg, can be generated following activation by the TCR, and are determined by the activation of certain transcription factors including T-box expressed in T-cells (T-bet), GATA binding protein-3 (GATA-3), RAR-related orphan receptor gamma (ROR- γ t) and forkhead box P3 (Foxp3), respectively (78). The strength of the TCR signal is also an important regulator of cell differentiation (91, 92). Post-translational modifications are able to influence the TCR signal strength, for example, citrullination enhances the production of a pathogenic form of helper cell, Th17 cells, through a low TCR signal (93). However, if the TCR is activated without the presence of co-stimulatory signals this will lead to the induction of tolerance via clonal anergy (94). After carrying out effector functions these cells will then undergo apoptosis to bring around homeostasis (95).

Some of the activated T-cells will go on to be memory T-cells which can last up to 20 years. These quiescent cells circulate the body but have the ability to rapidly respond to previously encountered antigen. These cells are heterogeneous and there are two key populations with different migratory and effector capacity, central memory (T_{CM}) and effector memory (T_{EM}). It is now thought the amount and type of cytokine present and length of stimulation determines the outcome of effector or central memory T-cells. T_{EM} have rapid effector function and reside in tissues whereas T_{CM} reside in lymph nodes with little effector function but can rapidly

differentiate into effector cells (Teff) (96). Memory cells are characterised by the downregulation of CD45RA with the simultaneous upregulation of CD45RO. T_{CM} and T_{EM} can be further identified by the differential expression of CD62L, which is expressed only on T_{CM} as it is required to aid migration.

1.2.1 Evidence of CD4⁺ T-cell Involvement in RA Pathogenesis

There is strong evidence suggesting that CD4⁺ T-cells play a crucial role in RA pathogenesis. The RA synovium has a high abundance of both CD4⁺ and CD8⁺ T-cells with a predominantly activated phenotype (80, 81, 97, 98). CD4⁺ T-cells can be found in high numbers in GCs and their presence depends on B-cell help (99). The level of infiltrating lymphocytes has been found to be higher in seropositive compared to seronegative disease though examination of the number of CD4⁺ T-cells in peripheral blood revealed no difference between the two disease groups (100, 101). The GCs in these patients were also shown to be less fibrotic and with a thinner synovial lining in seropositive disease.

The ability of T-cells to recognise autoantigens has long cemented their role in disease pathogenesis. Although no disease specific autoantigen has been discovered T-cells have been shown to activate in an antigen-specific manner to CII, gp39 and heat shock proteins (63, 102). The clonal expansion of identical TCRs found in distinct joints suggests a role for these in disease mediation (103).

Circulating CD4⁺ T-cells present in RA patients have been shown to have a highly altered phenotype compared to healthy controls. They have a high number of memory cells present in the joint but have also been shown to have a population of terminally differentiated cells (T_{EMRA}) which are both CD45RA⁺ and CD45RO⁺ (104). CD4⁺ T-cells have also been shown to have increased expression of cell surface makers programme cell death protein 1 (PD-1), CD25, CTLA4 and decreased CD69, OX40 and IL-2 (105). These cells have a markedly contracted TCR repertoire with high abundance of or absence of certain TCR- β chains and this has been shown to occur even at the earliest stages of disease (106). This occurs in both the naïve and memory cell repertoire suggesting defects in the generation of these cells. The analysis of T-cell receptor excision circles (TRECs) in RA reveals a decreased number compared to healthy controls. (107, 108). TRECs are small circles of deoxyribonucleic acid (DNA) which occur during TCR gene rearrangement in the thymus. TRECs are only present in T-cells which have come from the thymus and hence are diluted as a consequence of T-cell proliferation during memory formation or naïve homeostatic proliferation (109). This provides further evidence of diminished thymic production or increased cell turnover in RA.

The homeostatic expansion of circulating naïve CD4⁺ T-cells, evidenced by diminished TREC, in the wake of thymic dysfunction may be responsible for premature aging of T-cells in RA, demonstrated by the decreased length of telomeres in these cells, most prominent in naïve CD4⁺ T-cells (106, 110). This suggests an increase in replication of these cells occurring very early in disease, even potentially pre-dating disease onset (107). Despite the suggested increased replication of these cells in RA, telomere length does not correlate with disease duration.

Despite the activated phenotype and their ability to respond to previous stimulation, many studies have shown that CD4⁺ T-cells isolated from RA patients have reduced proliferation in response to stimulation. Hypo-responsiveness can also be seen in response to phytohaemagglutinin (PHA) and anti-CD3 and anti-CD28 stimulation with both a decrease in the number of responsive cells and a decrease in the magnitude of response (111). Proliferation of RA CD4⁺ T-cells is also impaired in response to autologous APCs, which could be partially restored by IL-2 (112, 113). Many of the observations of diminished responses to stimulus amongst RA CD4⁺ T-cells have been made in the context of established disease. One study has reported that undifferentiated arthritis (UA) patients who progress to RA display decreased numbers of cells dividing and a decreased number of cell divisions compared to UA patients who progress to non-RA diseases (114). Furthermore, synovial memory cells have impaired response to mitogen stimulation compared to peripheral blood cells (115). Expression of the TCR was found to be unaltered in RA, however, it was seen that cells had impaired Ca²⁺ responses indicating defects in downstream signalling which was attributed to decreased tyrosine phosphorylation of TCR- ζ chain (116-118). Furthermore, RA CD4⁺ T-cells have been shown to be resistant to apoptosis resulting in the perpetuation of these cells in the joints (119, 120).

Despite the hypo-proliferative phenotype of CD4⁺ T-cells documented in RA, many other studies have in fact shown that these cells appear to be *hyper*-proliferative and committed to pro-inflammatory effector lineages. In particular, naïve CD4⁺ T-cells have been shown to be dysregulated in RA, with a shift in glucose metabolism from glycolysis to the pentose phosphate pathway leading to lower levels of ATP, lactate and intracellular ROS in addition to insufficient activation of cell cycle kinase ATM (121). These features also result in a bias towards inflammatory Th1 and Th17 lineages and promotion of pro-inflammatory properties of T-cells (122). On the other hand, memory CD4⁺ T-cell hyper-activation has been documented to be due to CD147 expression and other features of CD4⁺ T-cells in RA include increased susceptibility to apoptosis and autophagy-deficiency (123, 124).

1.2.2 Roles of CD4⁺ T-helper cells in RA

CD4⁺ T-cells are important cells of the immune system that provide a “bridge” between innate and adaptive immunity. Differentiation of naïve CD4⁺ T-cells occurs following stimulation via the TCR, by cognate antigen presentation via the MHC and co-stimulation by APCs, and is greatly influenced by the presence of APC-derived cytokines. This results in activation of STAT signalling pathways, leading to changes in the chromatin of subset specific transcription factors including T-bet, GATA-3, ROR- γ t and Foxp3 resulting in the differentiation of Th1, Th2, Th17 and regulatory T-cells (Tregs) respectively, summarised in *Figure 2* (78). These master transcription factors can then exert control over transcriptional programs by modifying chromatin structure. The duration of antigenic stimulation and strength of TCR signal determines the outcome of effector T-cells (89, 125).

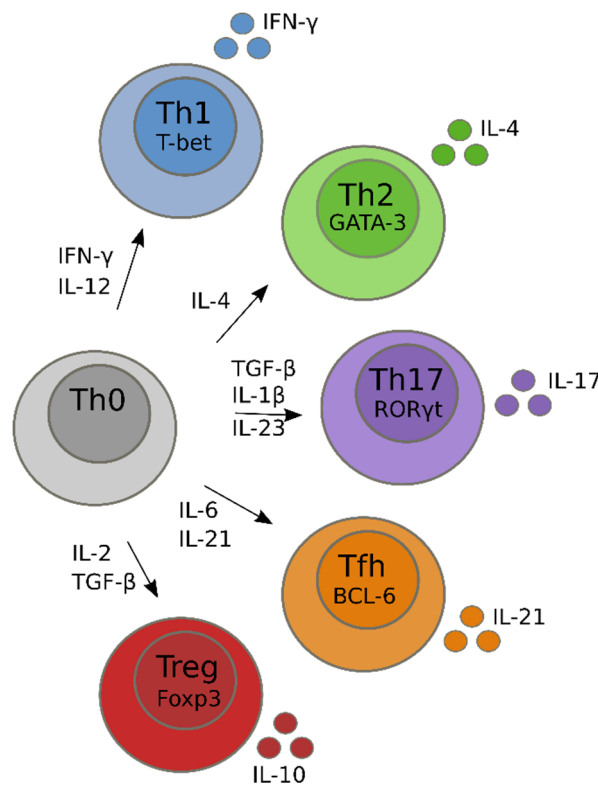


Figure 2. Overview of CD4⁺ T-helper subsets. Naïve CD4⁺ T-cells are able to differentiate into a number of T-helper subsets in the presence of particular cytokines and can be identified by their expression of master transcription factors and their production of cytokines.

1.2.2.1 RA as a Th1-mediated disease

RA was originally thought to be a Th1 mediated disease due to the high levels of IFN- γ detected in the synovial joints of established RA (126, 127). IFN- γ is the predominant cytokine produced by Th1 cells whose major role is in the cytotoxic response to extracellular bacteria. The majority of T-cell clones from the RA synovial membrane have been shown to represent Th1 cells and

are very stable with high levels of IFN- γ production (128). In contrast, the Th2 clones found were unstable and readily returned to the Th0 phenotype (129). Despite increased IFN- γ in the joints the production of this cytokine from peripheral T-cells of RA patients was shown to be reduced compared to healthy controls indicating that in contributing to synovial inflammation Th1 cells may become depleted from peripheral blood (130).

Other studies also point to blunted Th2 responses in established RA (131). Th2 cells are the key cell type directed against extracellular parasites by the production of IL-4, IL-5 and IL-13. T-cell clones from RA patients do not produce IL-4 on antigen challenge and RA CD4⁺ T-cells seem to have impaired Th2 differentiation under polarising conditions (132). The apparent imbalance in the Th1:Th2 ratio has been shown to correlate with disease activity, which is most prominent in early disease (127).

The fact that pregnancy, itself associated with muted Th1 immunity, leads to improved symptoms of RA in around 75% of patients (with up to 90% relapsing within 6 months post-partum (133)), has been invoked as further support for the concept of RA as a Th1 mediated disease. Those with allergic diseases also have reduced incidence of RA, which may be associated with the increased Th2 responses in these diseases, suggesting a possible beneficial role of Th2 cells (134). Further support for the protective role of Th2 responses in RA comes from the study of helminth infections, which have been shown to lead to the inhibition of autoimmune arthritis in two independent mouse models. This protective effect was shown to be dependent upon the presence of Th2 cells and specifically the IL-4 and IL-13 mediated STAT-6 pathway (135).

Some studies of RA have shown there is a predominance of Th2 related cytokines, including IL-2, IL-4, IL-13, IL-17 and IL-15, expressed by synovial T-cells in the earliest stages of disease whereas established disease is characterised by the presence of IFN- γ , TNF- α and IL-10 but low levels or absence of IL-2, IL-4, IL-5 and IL-13 (136). This may reflect the promotion of autoantibody production early in the disease. It is not clear whether this transient Th2 response in early disease represents a failed attempt at regulation or is merely a reflection of humoral immunity driving epitope spreading of autoantibodies. Despite the high levels of IFN- γ reported in the SF of new-onset RA patients the levels of IFN- γ in peripheral blood mononuclear cells (PBMC) from active RA patients have been shown by others to only reach significance with high DAS28, suggesting Th1 cells play a role late in the progression of RA (137). This indicates the transition to chronicity may occur during the switch from Th2 to Th1 predominance.

1.2.2.2 RA as a Th17-driven disease

Following the discovery of the Th1 and Th2 subsets a further subset of pro-inflammatory CD4⁺ T-cells were identified, Th17 cells. These cells defend the body from extracellular bacteria and are characterised by the production of IL-17, which has been shown to be elevated in a number of autoimmune diseases including RA. IL-17 can be detected in high levels in SF and ST along with high expression of its receptors (138-140). The overexpression of IL-17 has been shown to exacerbate joint destruction through its ability to promote the production of IL-1 and TNF- α from monocytes and production of IL-6, IL-8, granulocyte colony-stimulating factor (G-CSF) and MMPs from fibroblasts (141, 142). This cytokine also induces the production of receptor activator of nuclear factor kappa B ligand (RANKL) promoting local inflammation and exacerbating joint destruction (139). Despite the high frequency of IL-17, the frequency of Th17 cells have been reported to be unaltered in RA (143). However, others have shown increased Th17 cells in peripheral blood of RA across all stages of disease suggesting its importance in RA progression (137). These controversies, along with disappointing results of clinical trials of drugs such as ustekinumab and secukinumab (targeting the IL-23/IL-17 “axis”) have tempered early enthusiasm for the notion of RA as a Th17-mediated disease (144, 145). It is now thought that the IL-23/IL-17 axis is particularly important prior to RA onset, in the pre-articular phase of disease, with mouse models showing that the IL-23 dependent activation of Th17 cells resulted in the regulation of the glycosylation profiles of plasma cells, mirrored in individuals with circulating autoantibodies prior to arthritis development (146). This suggests a more prominent role for this axis in pre-RA, in particular in the development of seropositive disease.

1.2.2.3 A role for regulatory T-cell dysregulation in RA pathogenesis

Recently CD4⁺ T-cells with the capacity to inhibit and downregulate immune responses have been identified. These regulatory T-cells, termed Treg cells, have been heavily studied in the context of autoimmune disease as loss of immunological tolerance may result from the failure of these cells to control autoreactive T-cell responses. These cells are CD25⁺ Foxp3⁺ and make up somewhere between 5-15% of all CD4⁺ T-cells in healthy peripheral blood (147), and have suppressive effects on the proliferation and cytokine production of effector T-cells (Teff). Despite a low proliferative capacity *in vitro* (148, 149) these cells have been shown to be highly proliferative *in vivo* (150).

On first inspection of these cells it was seen that frequency is related to disease activity, however, further investigation into the abundance of these cells in the circulation reveals inconsistent findings with some showing increased frequency and others showing decreased or

similar frequencies (151-153). Despite these inconsistencies, it appears Treg cells are elevated in inflamed ST and SF compared to peripheral blood in RA, suggesting recruitment to the joint (152, 154). As these cells are not consistently decreased in RA, it was suggested instead that these cells had impaired function in RA (155). This was shown not to be the case and in fact, synovial Tregs have been noted to have increased suppressive abilities compared to peripheral blood (156). As no defects in Treg frequency and function have been confirmed early hints indicate that there may be a role instead for T_H17 resistance to Treg suppression, with studies showing that certain cytokines, such as IL-2, IL-7 and TNF, are able to abrogate Treg suppression in established chronic RA (157, 158). The abrogation of Treg suppression induced by TNF occurs despite the ability of monocyte bound TNF to expand functional Treg cells enhancing the regulatory properties of this cytokine (159).

1.2.2.4 Additional T-helper subsets in RA

Further subsets of CD4⁺ T-helper cells have now been identified including T-follicular helper (T_{fh}), T_H9 and T_H22 cells.

T_{fh} cells develop from naïve CD4⁺ T-cells and high affinity TCR interaction and have the capacity to regulate survival, proliferation, affinity maturation and differentiation of B-cells (160). Due to their crucial role in B-cell responses, these cells are present in high numbers in lymphoid tissue and aid in the formation and maintenance of GCs. These cells are characterised by the expression of C-X-C Motif Chemokine Receptor 5 (CXCR5), Inducible T-cell co-stimulator (ICOS), CD40L and PD-1 and by the production of IL-21, which have all been described to play roles in B-cell function and GC formation (161). Differentiation of these cells is promoted by IL-6 and IL-21 signalling in a STAT-3 dependent manner and these cytokines have been shown to induce the master transcription factor BCL-6 (162, 163). More recently it has also been shown that BCL-3, a gene highly expressed in RA patients and shown to be upregulated by IL-6 signalling, plays a role in development of T_{fh} cells by the upregulation of BCL-6 (164).

The presence of T_{fh} cells has been suggested as a biomarker for many diseases including RA, multiple sclerosis (MS), systemic lupus erythematosus (SLE), inflammatory bowel disease (IBD) and type 1 diabetes (T1D) (165). Increased frequency of circulating T_{fh} in the peripheral blood of RA has been documented and this level positively correlated with presence of anti-CCP antibody (166). Further evidence of their role in disease comes as IL-21^{-/-} deficient mice develop less severe spontaneous arthritis in the K/BxN model, with a reduced number of T_{fh} cells (167).

Th9 cells appear to play a role supplementary to Th2 against extracellular infections. A role for Th9 cells in inflammatory diseases such as experimental autoimmune encephalomyelitis (EAE) and IBD has been suggested, however, their role in rheumatic disease is unknown, with a possible role in T-cell dependent B-cell differentiation and antibody production (168). IL-9, the key cytokine of these cells, is increased in RA sera, SF and ST and levels correlate with the degree of inflammatory infiltrate (169, 170). IL-9 production by type 2 innate lymphoid cells (ILC-2s) has been shown in mouse models of arthritis to be crucial in cartilage destruction and bone loss. However, others have shown that the treatment of mice with this cytokine in chronic arthritis resulted in the resolution of inflammation, providing evidence that IL-9 may have a dichotomous role in different phases of disease. This suggests a novel therapeutic approach in the treatment of RA, where patients with active RA exhibit high levels of IL-9 producing ILC2s (171). Th22 cells produce IL-22 whose receptor is restricted to stromal cells, implicating Th22 cells in the interaction between the immune system and non-hematopoietic cells (172). Th22 responses show many similarities with the Th17 response and their increased presence in RA has been positively correlated with the levels of Th17 cells, however, so far no pathologic associations of Th22 cells with RA have been reported (173).

1.2.2.5 Plasticity of T-helper cell effector function

The T-helper cell subsets have been shown to have a considerable degree of plasticity. Following stimulation with IFN- γ and to a lesser extent IFN- α upon viral infection Th2 cells can be induced to express Th1 transcription factor T-bet, with considerable overlap in their cytokine production (174). Th17 cells have a highly transient nature and T-cells with a mixed Th1/Th17 phenotype have been detected in the gut of patients with Crohn's disease (CD), reported to arise from modulation by the presence of IL-12 (175). Cells with a Th17/Th2 profile have been found in patients with chronic asthma which can be derived by the culture of Th17 cells with IL-4 (176).

Treg cells have also been shown to have a degree of plasticity. The Treg transcription factor Foxp3 can bind ROR α , a transcription factor with complementary roles to ROR- γ t in the differentiation of Th17 cells, suggesting a common precursor for both subsets and the balance of cytokine influences their subsequent differentiation (177). Numerous cytokines have been implicated in driving the conversion of Tregs to Th17 cells, including IL-6, transforming growth factor- β (TGF- β), IL-21, IL-23, IL-1 β , IL-2 (178-180). Tregs which have been induced to convert to Th17 cells produce high levels of IL-17 with high expression of ROR- γ t, however, these cells still have the ability to suppress proliferation (181, 182).

Therefore, a simplistic view of differentiated T-helper subsets as rigidly discrete entities is now recognised to be unlikely. A more nuanced reality, in which dynamic effector functions of these cells are evident over time according to shifting immune contexts, presents challenges in investigating the pathogenesis of various autoimmune disorders.

1.2.3 Mouse Models Implicating CD4⁺ T-cells in RA

Mouse models provide a significant contribution to the understanding of the molecular mechanisms behind disease pathogenesis. There are numerous models used in the investigation of RA including; CIA, antigen induced arthritis (AIA), the SKG mouse and the K/BxN mouse (183). Many of these models are notable for their dependence upon T-cells during disease initiation and maintenance.

1.2.3.1 Collagen Induced Arthritis

The CIA model is the most extensively studied model of autoimmune inflammatory arthritis. This uses the immunisation of mice with CII, a protein found in cartilage, in the context of adjuvant to induce polyarticular arthritis akin to RA (184). Similarly, as with human RA, susceptibility to CIA is also associated with MHC class II, providing strong support for this model's relevance to human disease for the purpose of mechanistic investigation. Humanised mice with the alleles DR4 and DQ8 have increased susceptibility to disease (185, 186). The elimination of CD4⁺ T-cells prevents disease and the transfer of these cells to severe combined immunodeficient (SCID) mice results in the transfer of disease (187, 188). In contrast to human RA, there is no presence of RF and in this model male mice are more susceptible to disease due to a protective role of hormones binding estrogen receptors (189).

The CIA model is predominantly Th1 driven in the pre-arthritic stage of disease, indicated by the high levels of IFN- γ production (190). Neutralisation of IFN- γ prior to disease onset has a protective role, however, neutralisation late in the disease course exacerbates disease suggesting a change in helper cell contribution to pathogenesis as in human RA (191). Th17 cells have also been shown to be important in this model with increasing synovial IL-17 mRNA levels as disease progresses (192). Knock out (KO) of the IL-17A gene results in a decrease in disease incidence and severity while blockade following onset reduced progression and joint damage (193). Furthermore, the elimination of Tregs increases disease onset and severity (194). The increased number and function of Tregs is associated with disease improvement and resistance to disease (195, 196).

1.2.3.2 Antigen Induced Arthritis

Intra-articular injection of mice with methylated bovine serum albumin (BSA) results in the induction of inflammatory arthritis in the AIA model. The role of CD4⁺ T-cells is highlighted in this disease as anti-CD4 can ablate disease, with more effect late in the disease (197). Blockade of the IL-17 receptor has also been shown to reduce disease severity implicating Th17 cells in the pathogenesis (198).

1.2.3.3 Models of Spontaneous Arthritis

Spontaneous models of RA result from mutations in key genes of the immune system. For example, spontaneous arthritis arises in the SKG model as a result of a point mutation in zeta chain associated protein kinase 70 (ZAP-70), a key molecule in TCR signal transduction, resulting in replacement of tryptophan with cysteine at position 489. This lowers TCR signalling and affects the process of selection in the thymus, resulting in the escape of autoreactive T-cells (199). This model is characterised by high levels of pro-inflammatory cytokines IL-6, IL-1 β and TNF- α which can drive Th17 responses (200). The ablation of IL-17 meant that disease could not be transferred to recombination activating gene 2 (Rag2)^{-/-} mice highlighting the dependence of this model on Th17 (201). Treatment with anti-CD4 protects against disease induction and progression by decreasing the numbers of Th17 cells and increasing the amount of Tregs (202).

The K/BxN model involves crossing of non-obese diabetic (NOD) mice, which are susceptible to autoimmune disease with TCR transgenic mice recognising the autoantigen glucose-6-phosphate isomerase (GPI) results in spontaneous joint disorder driven by CD4⁺ T-cells. The deletion of IFN- γ R and the IL-12p35/IL-12RB2 genes results in reduced levels of arthritis indicating a role for Th1 cells in the disease (183). The deletion of Tregs results in faster progression of disease with more severe joint destruction highlighting their importance in disease models (183, 203).

Such a large number of arthritis models with wide ranging mechanisms of disease highlights the importance of T-cells in the pathogenesis of various models that share the inflammatory arthritis phenotype and hence make a compelling case for their contribution to human inflammatory arthritis.

1.2.4 RA Therapies Implicating CD4⁺ T-cells

Many therapies used in the treatment of RA have been shown to have significant effects on CD4⁺ T-cell number and function, supporting a role for these cells in disease pathogenesis.

Disease modifying anti-rheumatic drugs (DMARDs) are the current first line therapeutics in the treatment of RA used to slow disease progression. These act by altering underlying disease processes, for example methotrexate (MTX) is an immune suppressant which works by inhibiting purine and pyrimidine synthesis ultimately blocking cell proliferation (204). This drug has been shown to decrease the production of IFN- γ and IL-2 by PBMC in vitro while promoting IL-4 and IL-10 production, modulating the balance between Th1 and Th2 (205). Those patients who achieve remission following MTX treatment have been shown to have increased frequency of naïve CD4⁺ T-cells at baseline, a potential biomarker of response (206).

Other DMARDs have also been shown to affect the Th1-Th2 balance such as Leflunomide and Sulfasalazine (SSZ). These drugs inhibit Th1 while promoting Th2 responses with SSZ specifically inhibiting the production of IL-12 and IFN- γ while increasing production of IL-4 (207, 208).

Despite the efficacy of conventional DMARDs the introduction of biologics, which target specific inflammatory mechanisms, has greatly advanced the treatment of RA. The first biologic developed was anti-TNF. Primarily directed towards macrophages and monocytes this therapy has indirect effects on CD4⁺ T-cells by modulating IFN- γ and IL-17 production to alter the frequency of Th1 and Th17 cells (209, 210).

Abatacept is a soluble Ig fusion protein of the CTLA-4 extracellular domain, able to bind co-stimulatory molecules CD80/CD86 with higher affinity than CD28, resulting in the blockade of CD4⁺ T-cell co-stimulation. Production of pro-inflammatory cytokines such as IFN- γ , IL-6, TNF- α and TGF- β has been shown to be reduced following treatment (211, 212). Increased response to treatment, reduced disease progression and improved mobility have all been documented following treatment with CTLA-4 (213). More recent studies have also shown that treatment with this modulator of co-stimulation may restore tolerance towards citrullinated antigens (214).

Despite many successful therapies whose mechanism of action involve modulating CD4⁺ T-cells number and function, antibodies directed against anti-CD4 have not been successful in the treatment of RA with such antibodies having moderate efficacy but high levels of toxicity (215, 216). This suggests the overall depletion of T-cells is not beneficial which may be due to the depletion of certain subsets such as Tregs, which may be beneficial in disease, therefore more complex modulation of T-cell subsets may be necessary in treatment of RA. Timing of treatments may also be important in the success of such therapies due to the differences in cytokine profiles throughout the progression of RA.

1.3 Cytokines in Early RA

Cytokines are the key regulators of inflammatory responses, a crucial component in the pathogenesis of autoimmune diseases. The presence of circulating cytokines is dysregulated in RA, occurring even before the clinical onset of RA, and the contribution of these cytokines in immune dysregulation in the pathogenesis of RA is now being elucidated, *Figure 3*.

Cytokines elevated prior to symptom onset include IL-6, TNF, IL-1 β , granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-4, IL-5 and IL-17. Their presence prior to disease onset highlights the importance of certain cytokines in disease initiation. For example, Th2 specific cytokines IL-4 and IL-5 show transient expression during the earliest stages of disease suggesting a discriminatory role for these cytokines in disease progression (136, 217). The IL-17/IL-23 axis also appears particularly important in the pre-clinical phases of disease, particularly seropositive disease, as changes in antibody glycosylation mediated by these cytokines has been shown to trigger arthritis in mouse models (146). Furthermore, identification of the interferon gene signature has been shown to predict development of RA in at risk individuals (218). Unfortunately, the blockade of IL-1, IL-17 and IL-23 have been unsuccessful in established disease, this may be due to their particular dominance in early stages of disease whereas trials of these drugs largely occurs in established disease (144).

In contrast, there is a dominance of pro-inflammatory cytokines TNF, IL-6 and GM-CSF during the inflammatory disease phase. TNF is responsible for the activation of osteoclasts and chondrocytes to drive joint destruction and induces the production of other pro-inflammatory cytokines including MMPs, IL-1 and IL-6 (219). Both TNF and IL-6 have been shown to abrogate PD-1 mediated suppression of CD4⁺ T-cells in inflammatory arthritis, highlighting these cytokines may be key mediators in the maintenance of synovitis (220). The blockade of these cytokines is highly successful in established disease, which may reflect their dominance in the later stages of disease pathogenesis.

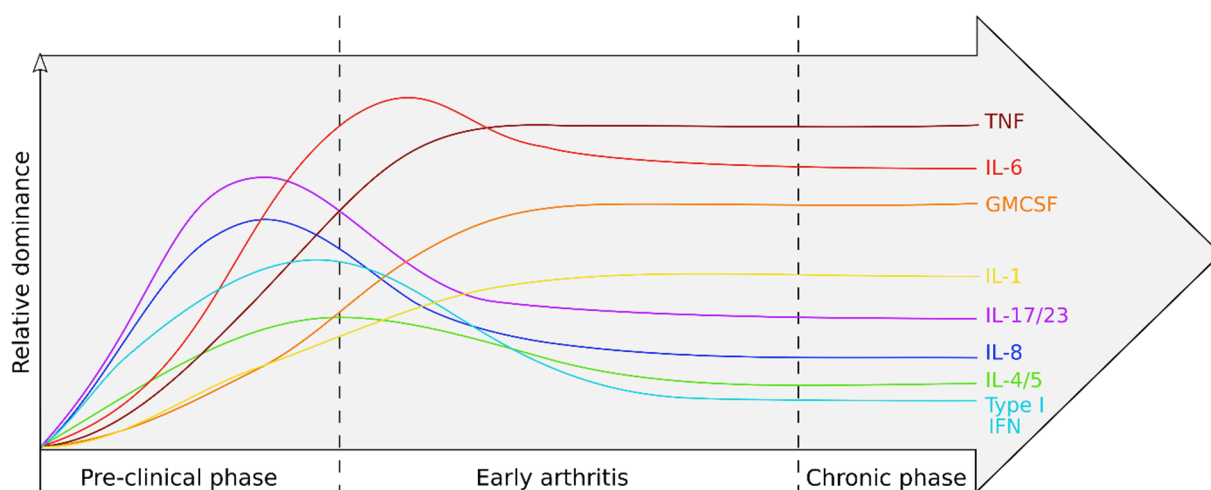


Figure 3. Cytokine dominance throughout the progression of RA. Individual cytokines show differing patterns of relative importance throughout the progression of seropositive RA. The dominance of cytokines throughout seronegative RA is yet to be fully elucidated. Figure adapted from Ridgley et al. *Current Opinion in Rheumatology*, 2017.

These studies suggest that the hierarchical dominance of individual cytokines alters during disease phase, and likely between individuals, with cytokine dominance in early stages of seronegative disease remaining elusive (221). This has consequences on the optimal timing of therapeutics and controlling the balance of these cytokines during different disease stages may be a key strategy in therapeutic targeting of RA.

1.3.1 IL-6

IL-6 is of fundamental importance as a pro-inflammatory cytokine in RA (222). IL-6 was first discovered in 1986 as a T-cell derived cytokine thought to play a key role in the differentiation of B-cells into antibody producing cells (223). It is now known that IL-6 is a pleiotropic cytokine with a wide range of functions on a number of cells and tissues. Human IL-6 is a molecule 184 amino acids in length, with 65% and 42% homology with murine cDNA and protein respectively (224). The protein is 21-28kDa and has a structure made up of four long α helices (225).

This cytokine belongs to the IL-6-type family along with IL-11, IL-27, IL-32, leukaemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), cardiotrophin-like cytokine (CLC), neurotrophin-1 (NNT-1) and neuropoietin (NPN). These cytokines are produced by APCs, T-cells, B-cells, monocytes and non-hematopoietic cells such as fibroblasts, keratinocytes, endothelial cells, mesangial cells, adipocytes and tumour cells (226). They are produced in response to stimuli such as IL-1, TNF- α ,

lipopolysaccharide (LPS) and platelet derived growth factor (PDGF) to activate differentiation, survival, apoptosis and proliferation.

1.3.2 IL-6 Receptors

Members of the IL-6 type cytokine family signal via the common signal transducing receptor chain gp130 (CD130), sometimes called gp130 family cytokines, providing functional redundancy within the system. Gp130 is a signal transducing receptor of 130kDa, which is ubiquitously expressed on cells including heart, kidney, spleen, liver, lung, placenta, brain and hematopoietic cells (227). Deletion of gp130 is embryonically lethal due to hypoplastic ventricular myocardium and reduced hematopoietic progenitors (228). The conditional deletion of gp130 results in defects in hematopoietic, cardiac, neurological, hepatic, pulmonary and immunological systems (229). On the other hand, continuous activation of this receptor causes hypergammaglobulinemia, splenomegaly, mesangial proliferative glomerulonephritis, lymphoid infiltration in the lung and myocardium hypertrophy (230).

Despite studies documenting the ubiquitous expression of gp130 this receptor has been shown to be regulated on some cells. Vascular smooth muscle cells express modest levels of gp130 which is upregulated in response to IL-6 driving an inflammatory state (231). Downregulation of gp130 expression also occurs in response to extracellular signal-regulated kinases 2 (ERK2) binding to the promoter region to inhibit expression. The p38 mitogen-activated protein kinase (MAPK) pathway also inhibits gp130 expression in juvenile idiopathic arthritis (JIA) patients to prevent excessive signalling of IL-6 (232, 233).

In addition to the β receptor common to all IL-6 family members these cytokines also have their own specific α receptor subunit. IL-6 cannot signal directly via gp130 and requires the presence of an additional signalling molecule, the IL-6R (also known as CD126). This is a type 1 transmembrane glycoprotein of 80kDa which contains an extracellular Ig-like domain, a cytokine receptor homology domain, a transmembrane domain and an intracellular domain which has no kinase activity (225).

Unlike the β subunit the expression of IL-6R is restricted to hepatocytes, neutrophils, monocytes, macrophages and lymphocytes allowing tight control of signalling (234). The deletion of the IL-6R results in many of the same inflammatory defects seen in IL-6 deficient mice namely defects in the generation of acute phase responses, impaired neutrophil resolution and delayed macrophage infiltration. However, differences do exist between the two KO systems with defects in wound healing less severe in IL-6R^{-/-} (235). The deletion of IL-6R specifically in hepatocytes also reveals lower insulin sensitivity and lower glucose tolerance

(236). Conditional deletion in hepatocytes also suggests these cell types are crucial for the production of IL-6R in the acute phase response (235).

In the absence of the membrane bound IL-6R IL-6 is able to signal via gp130 by enlisting a soluble form of the α receptor (sIL-6R). This soluble form of the receptor lacks both transmembrane and cytoplasmic regions and is generated by the proteolytic cleavage of membrane bound IL-6R or by the translation of an alternatively spliced IL-6R messenger ribonucleic acid (mRNA) (237, 238). Differential splicing of the IL-6R results in a 50-55kDa transcript lacking the 94 base-pairs which encode the transmembrane domain of the receptor. This has been shown to be the predominant form of the sIL-6R with levels decreasing with age suggesting a switch to generation by proteolytic cleavage (239). The cleavage site of the IL-6R is found immediately prior to the transmembrane domain and shedding occurs via cleavage carried out by the a disintegrin and metalloprotease domain 10/17 (ADAM-10/17), also known as tumour necrosis factor α converting enzyme (TACE) (240). It has been shown that ADAM10 causes slow and constitutive shedding compared to ADAM17, which causes rapid proteolysis upon activation (241). IL-6R shedding has been shown to be induced upon phorbol 12-myristate 13-acetate (PMA) stimulation, by pro-inflammatory cytokines, bacterial toxins, Ca^{2+} , C-reactive protein (CRP) and by apoptosis (242-247). The shedding of the IL-6R is a rapid process unlike alternative splicing which is a slower process hence subject to less control.

The main producers of sIL-6R have been identified as hepatocytes and hematopoietic cells as the deletion of IL-6R in each cell type led to a decrease in the serum level of sIL-6R by 32% and 60% respectively (235).

1.3.3 Classical versus Trans Signalling

Signalling via gp130 and the membrane bound IL-6R complex is called classical signalling. During this signalling mechanism IL-6 binds directly to the membrane bound IL-6R which is then able to recruit gp130 subunits to coordinate signalling. Despite being the receptor specific for IL-6, CNTF and IL-27 have also been demonstrated to bind the IL-6R in vitro, although with a much lower affinity than IL-6 (248, 249).

The sIL-6R is able to bind IL-6 with a similar affinity to the membrane bound IL-6R of 0.5-2nM and signalling via this mechanism is termed trans signalling (239). During this mechanism, IL-6 first binds sIL-6R in the extracellular matrix to create the IL-6/sIL-6R complex, which is then able to bind to gp130 to initiate signalling. This method of signalling is agonistic therefore is able to sensitise cells not expressing the IL-6R to IL-6, broadening cellular responsiveness and prolonging the effective half-life of the cytokine.

Recently a further method of IL-6 signalling has been reported termed “IL-6 cluster signalling”. This method of signalling involves the trans presentation of IL-6 bound to IL-6R on DCs to T-cells during cognate interactions (250). This method of signalling was shown to be crucial in the generation of pathogenic Th17 cells in vivo. The various mechanisms of IL-6 signalling are summarised in *Figure 4*.

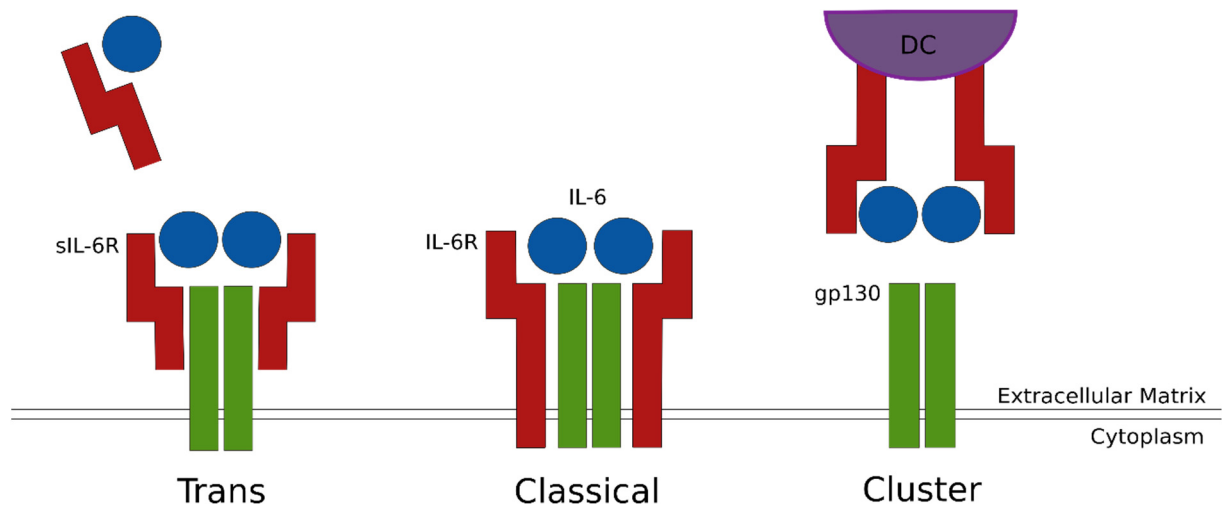


Figure 4. Signalling mechanisms of IL-6. IL-6 can signal via three distinct mechanisms; classical signalling that requires membrane bound IL-6R, trans signalling which requires soluble IL-6R and newly described cluster signalling where IL-6 bound to IL-6R on DCs is presented to T-cells.

The signalling of IL-6 is made more complex by the presence of soluble forms of the gp130 receptor, sgp130 (251). These soluble receptors are generated by alternative splicing and can be detected at high levels in circulation, around 100-400ng/ml (252, 253). Several forms have been detected at 50, 90 and 110kDa, which likely arise from differential processing. One of these forms is sgp130 rheumatoid arthritis associated peptide (sgp130-RAPS) (254). This soluble form of gp130 binds the IL-6/IL-6R complex in circulation to specifically inhibit trans-signalling (255). Levels of sIL-6R and sgp130 in the steady state are 1000 times higher than IL-6 levels suggesting in healthy setting IL-6 is released which binds to sIL-6R and subsequently to sgp130 to provide a method of neutralisation.

The contribution of both the classical and trans signalling pathways in various diseases has been assessed indicating the preferential involvement of the trans signalling pathway in numerous diseases. Classical signalling is crucial in the generation of the acute phase response (APR) hence, this pathway has been suggested to be responsible largely for the regenerative properties of IL-6. Confirming this, the classical signalling pathway has been shown to be an important factor in the elimination of pathogens, including *Listeria monocytogenes* and *Mycobacterium tuberculosis* (256, 257).

In contrast, despite the beneficial role of trans signalling in the rapid development of cytotoxic CD8⁺ T-cells against viruses and intracellular bacteria, numerous studies highlight the pro-inflammatory role of this signalling mechanism in disease (258). The trans signalling pathway has been shown to be required for the progression of pancreatic adenocarcinoma and promotes pancreatitis associated lung injury (259, 260). The specific blockade of trans signalling with sgp130Fc, a dimer of two gp130 molecules fused to an Fc domain of an IgG protein, has been shown to be beneficial in animal models of disease including IBD, coronary artery disease and malaria (261-263). Evidence also suggests the involvement of this signalling mechanism in inflammatory arthritis as Hyper-IL-6, a fusion protein of the IL-6/sIL-6R complex, restores disease activity in AIA whereas sgp130Fc was able to ameliorate disease in this model and in a model of CIA (264, 265). These data highlights the importance of the trans signalling pathway in the pro-inflammatory effects of IL-6.

1.3.4 Intracellular IL-6 Signalling

In the initiation of signalling IL-6 first binds the membrane bound or soluble IL-6R and this complex of IL-6-IL-6R can then associate with gp130 inducing dimerization to form a hexameric structure (225). The binding of IL-6 to its receptor leads to rapid internalisation mediated by gp130. This process forms a high affinity functional receptor complex which is able to initiate signal transduction. The Janus kinase family are tyrosine kinases which transduce signals mediated by the IL-6 family cytokines, including JAK1, JAK2 and TYK2 (266). These molecules are constitutively associated with the cytoplasmic tail of gp130 by multiple docking sites and are moved into close proximity upon dimerization. Upon JAK activation 4 specific tyrosine residues in the cytoplasmic domain of gp130 are phosphorylated and these become docking sites for transcription factors such as STAT-3 and to a lesser extent STAT-1 (267).

1.3.4.1 STAT-3

Following the recruitment of STATs these molecules are then phosphorylated by JAKs at a single tyrosine residue, Tyr701 for STAT-1 and Tyr705 for STAT-3, to induce dimerization (268). This can produce heterodimers, but more commonly homodimers which are able to translocate to the nucleus and initiate the transcription of certain genes containing STAT responsive elements. Although the mechanism of entry into the nucleus is largely unknown STAT-3 has been shown to bind the glucocorticoid receptor (GR) which contains nuclear localisation sequences (NLS) providing a possible mechanism of entry (269, 270).

STATs are a family of transcription factors with Src homology 2 (SH2) domains that are induced as a result of cytokine signalling. Their translocation to the nucleus allows the binding

to responsive elements to promote transcription. STAT-3 can be activated by phosphorylation at either tyrosine or serine sites Y705 or S727, respectively (271). Tyrosine phosphorylation is mediated by JAK1 and is required for dimerisation and subsequent translocation to the nucleus. Serine phosphorylation is mediated by several kinases including ERK1/2, p38 and c-Jun N-terminal kinases (JNK) depending on the signal and occurs in MAPK consensus site to both positively and negatively control gene transcription (272).

1.3.4.2 Other signalling pathways

IL-6 can also to a lesser extent initiate the MAPK and phosphoinositide-3 kinase (PI3K) pathways (273, 274). These pathways are important in mediating cell growth and cell cycle and have been shown to be important mediators of the anti-apoptotic effects of IL-6. In the case of MAPK, tyrosine phosphatase SHP2 is recruited to the Y759 phospho-tyrosine residue on gp130 leading to phosphorylation which promotes the interaction with growth factor bound protein 2 (Grb2) which is associated with Son-of-Sevenless (SOS) or Grb2-associated binder 1 (GAB1). Signals are then transmitted to activate ERK-MAPK resulting in the activation of transcription factors such as NF-IL-6 (C/EBP β) and serum and glucocorticoid-stimulated kinase (SGK) (275, 276). In the PI3K pathway, protein kinase B (Akt) is phosphorylated at serine/threonine sites which enables it to phosphorylate downstream molecules such as B-cell lymphoma 2 (BCL2) family member BCL2-associated death promoter (BAD). This molecule then associates with 14-3-3 sequestering form B-cell lymphoma-extra-large (BCL-xL) to promote cell survival (225). Despite the documented roles of MAPK and PI3K in IL-6 signalling the predominant pathway activated by IL-6 is the STAT-3 pathway which has been shown to be crucial for cell survival and cell cycle transition, *Figure 5*.

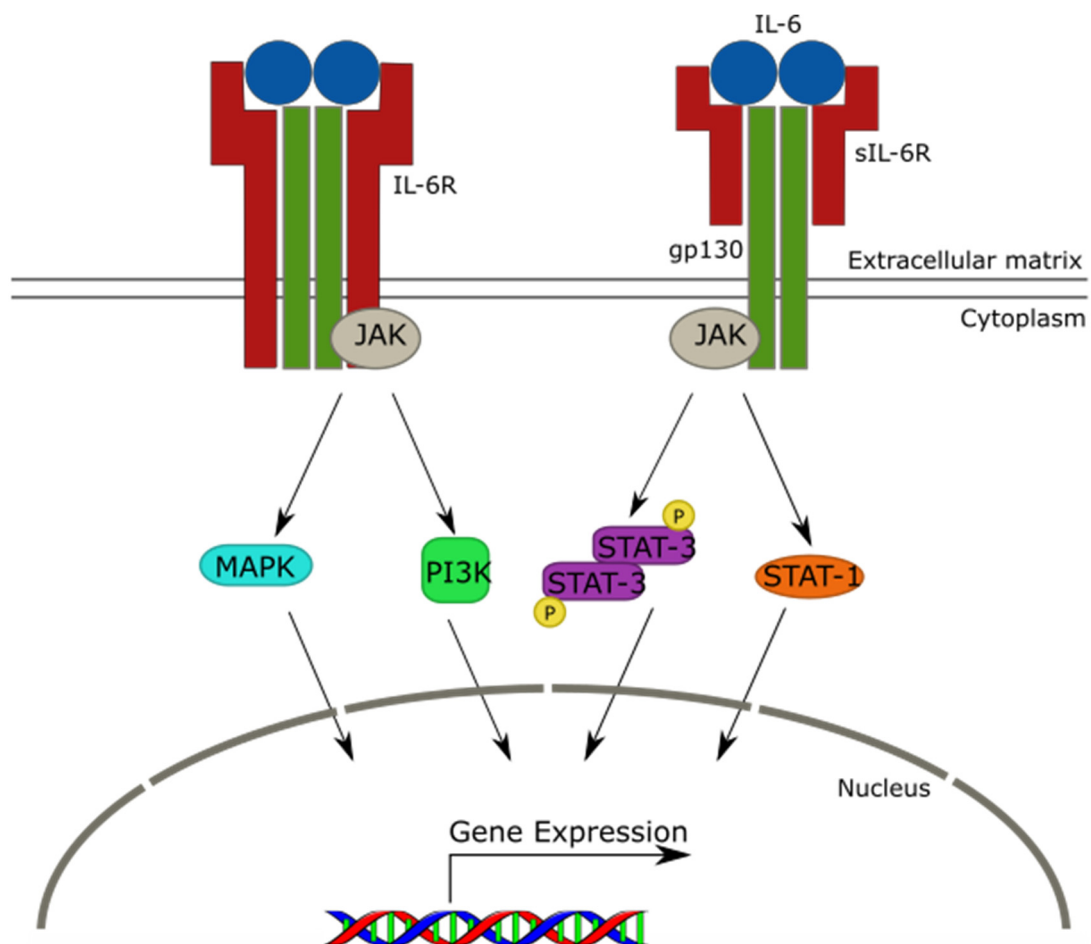


Figure 5. Downstream signalling mechanisms of IL-6. Following the binding of IL-6 to its receptors JAK molecules are activated which leads to the phosphorylation of gp130 and subsequently initiation of downstream signalling pathways such as MAPK, PI3K and STAT. The predominant signalling pathway of IL-6 is the STAT-3 pathway, where STAT-3 molecules are phosphorylated, form homo-dimers and translocate to the nucleus to induce gene expression.

1.3.5 Signal Termination

Termination of signalling via the IL-6-STAT-3 signalling pathway can be achieved by multiple mechanisms. One of these mechanisms involves the protein tyrosine phosphatases (PTP) which are able to dephosphorylate components of signal transduction to prevent the recruitment or activation of STAT molecules. SHP2 is a key PTP which is recruited to Tyr759 on the SH2 domain of activated gp130. This leads to protein unfolding and reduced enzymatic activity (277). SHP2 can also bind JAK and STAT molecules providing several means of signal termination. SHP2 regulates both the amount of activity and the amplitude of activity of STAT3 but also has a positive role in MAPK signalling (278). Other PTPs involved in the termination of IL-6 signalling include PTP1B, CD45, SHP1 and PTPeC, which alters the onset of STAT-3 activation (279-281).

The JAK/STAT signalling pathway is also regulated by protein inhibitors of activated STAT (PIAS). This family of molecules contains 5 molecules which interfere with DNA binding of STATs in order to prevent gene expression (282). PIAS1 specifically inhibits STAT-1 signalling whereas PIAS3 is specific for inhibition of STAT-3 (283, 284). Interaction requires STAT tyrosine phosphorylation, although the mechanism of inhibition remains unclear.

The final mechanism of inhibition of signalling involves the cytokine inducible SH2 proteins (CIS) or the stat inducible stat inhibitors (SSI). These consist of janus kinase binding protein (JAB) and suppressor of cytokine signalling 1-7 (SOCS1-7) which all contain SH2 domains and in the case of SOCS include a SOCS box. These SOCS are classical feedback inhibitors as they are induced by JAK/STAT signalling. SOCS1 and SOCS3 are inhibitors of IL-6 signalling and are able to inhibit phosphorylation of gp130, JAK and STAT. SOCS3 associates with Tyr759 in activated gp130 which is also the binding site for SHP2, although SOCS3 can bind with higher affinity (285).

A further mechanism of regulation involves the regulation of IL-6 and its receptors. The availability of IL-6 can be regulated at the level of mRNA or protein. The p38 MAPK signalling pathway has been shown to play an important role in stabilisation of IL-6 mRNA by the activation of mitogen activated protein kinase activated protein kinase 2 (MK2) (286). Alternatively, IL-6 protein can be regulated by protein proteolysis. For example, at sites of inflammation IL-6 induces serine protease elastase, proteinase 3 and cathepsin G from neutrophils which in turn degrade IL-6 (287). A widespread mechanism for signal termination is receptor internalisation. It has been shown that the regulation of IL-6 in hepatocytes involves down-regulation and degradation of gp130, suggesting gp130 plays an important role in preventing over activation (288). This was further validated and suggested to be due to gp130 di leucine motif (289). It has since been suggested that gp130 internalisation is independent of signalling as cells not capable of activating the JAK/STAT molecules are still efficiently endocytosed (290).

1.3.6 IL-6 Functions in Health and Disease

IL-6 is induced rapidly by infection or tissue injury in the host defence mechanism. IL-6 is primarily regarded as pro-inflammatory although it also has anti-inflammatory properties, largely carried out by the classical signalling pathway (253). The classical signalling pathway is involved in homeostatic processes such as the acute phase response, glucose metabolism and haematopoiesis whereas the trans signalling pathway is involved in activation of stromal tissue, maintenance of effector T-cell functions and recruitment and apoptosis of leukocytes (291).

The functions of IL-6 are context dependent and affect cells of the immune system in addition to non-immune cells.

1.3.6.1 Transition from Acute to Chronic Inflammation

During the acute inflammatory response, IL-6 has an anti-inflammatory role by controlling the magnitude of acute inflammation by suppression of pro-inflammatory cytokines and stimulating the production of anti-inflammatory mediator IL-1R antagonist (292, 293).

IL-6 and sIL-6R signalling has been shown to be a key regulator in the transition from acute to chronic inflammation by aiding the switch from neutrophil to monocyte recruitment, thereby assisting the resolution of inflammation (294). The proteolytic cleavage of IL-6R on infiltrating neutrophils drives the process of trans signalling in resident tissue cells causing suppression of neutrophil attracting chemokines, such as CXCL1 and CXCL8 enhancing trans migration of neutrophils away from inflammatory sites and contributing to apoptosis via caspase-3 (295, 296). IL-6 enhances monocyte chemotactic protein 1 (MCP-1) and cell adhesion molecules such as intracellular adhesion molecule 1 (ICAM-1) on endothelial cells and CD62L on lymphocytes to aid recruitment of monocytes and leukocytes (294, 297).

During the chronic inflammatory response IL-6 is responsible for inducing cellular responses such as plasma B-cell differentiation, antibody production and T-cell activation (298). IL-6 has the ability to skew monocyte differentiation to macrophages by the upregulation of MCSF expression and in DCs IL-6 can inhibit NF- κ B, impairing their migration and T-cell stimulatory ability (299, 300).

These data highlight the role of IL-6 signalling in chronic inflammation. In contrast to acute inflammation in the chronic inflammatory response IL-6 has a pro-inflammatory role favouring the accumulation of cells and promoting their survival, ultimately leading to tissue damage.

1.3.6.2 Acute Phase Response

The APR is induced by inflammation to minimise tissue damage and consists of fever and increased vascular permeability. Acute phase proteins (APPs) are produced mainly by hepatocytes and the presence of IL-6 has been shown to regulate the production of these APPs. IL-6 has been shown to increase the production of positive APPs such as serum amyloid A (SAA), haptoglobin, alpha 1 acid glycoprotein (α 1AGP), CRP, antitrypsin and fibrinogen and decrease the production of negative APPs, including transferrin and albumin (301, 302). The APR and hence IL-6 is necessary for the recovery from inflammation. A further role of IL-6 in the mediation of inflammation is the induction of excessive production of vascular

endothelial growth factor (VEGF) by synovial and mesothelial cells contributing to enhanced angiogenesis and vascular permeability (303, 304).

1.3.6.3 Iron Metabolism

IL-6 is also involved in the regulation of iron by the induction of iron regulatory hormone hepcidin (305). This blocks the action of iron transporter ferroportin-1 preventing the absorption of iron. The consequence of this inhibition is the development of hypoferremia and anaemia, which is often observed in chronic inflammatory diseases, including RA. The severity of this anaemia has been shown to correlate with serum IL-6 levels (306).

1.3.6.4 Lipid Metabolism

A role for IL-6 in controlling metabolism has also been suggested. IL-6 is produced by adipocytes and levels are found to be elevated in obesity, correlating with the subsequent development of type 2 diabetes mellitus (T2DM) (307, 308). Further studies have shown IL-6 correlates with increased fat mass and can increase insulin stimulated glucose disposal in healthy individuals (309). IL-6 KO mice develop late onset obesity with glucose intolerance, insulin resistance and hepatic inflammation, however; exposure to a high fat diet corrects obese phenotype although hepatic inflammation and glucose intolerance persists (310, 311). In contrast, IL-6 is also produced by skeletal muscle cells upon exercise. This promotes glycogenolysis and lipolysis in skeletal muscle to enhance muscle efficiency and muscle growth highlighting the complex role of this cytokine in metabolism (312).

1.3.6.5 Cartilage and Bone Metabolism

The metabolism of cartilage and bone is also influenced by IL-6 trans signalling. Signalling via the sIL-6R stimulates the production of RANKL, a molecule responsible for osteoclast formation leading to bone resorption (313). Classical signalling of IL-6 inhibits the insulin-induced differentiation of chondrogenic progenitor cells and increases MMP production of chondrocytes (314, 315). On the other hand, trans signalling induces the production of tissue inhibitors of MMPs (TIMPs) from chondrocytes suggesting a role for both signalling mechanisms in the contribution of matrix turnover (316).

A number of processes are also mediated by trans signalling. IL-6 has been shown to be an important mediator of liver regeneration as IL-6 deficient mice have impaired regeneration (317). This process is dependent on the presence of sIL-6R, which is necessary for hepatocellular proliferation (318). Finally, trans signalling has been shown to be important in the sensation of pain. The sIL-6R signalling pathway plays a role in sensitising nociceptors to heat, mediated by protein kinase C (PKC) δ and the PI3K pathway (319, 320).

1.3.7 STAT-3 Functions in Health and Disease

STAT-3 is the major signalling molecule downstream of IL-6 family cytokines and gp130 signalling. The ablation of STAT-3 leads to early embryonic lethality suggesting a crucial role of this transcription factor in development (321). The conditional deletion of STAT-3 has led to the discovery of a wide range of diverse roles of STAT-3 in specific cells types. Deletion of STAT-3 has been shown to impair keratinocyte and endothelial cell migration and depletion in thymic epithelial cells results in a loss of thymocytes and thymic architecture (322, 323). STAT-3 has been implicated in the differentiation, and subsequent proliferation of myeloid cells into macrophages (324). This is confirmed as defective STAT-3 in myeloid cells leads to aberrant expression of MHC II molecules and increased production of anti-inflammatory cytokines (325). STAT-3 has been shown to induce the expression of many genes in the cell cycle including Fos, Cyclin-D, c-Myc, proto-oncogene serine/threonine- protein kinase (PIM-1) as well as genes involved in protection from apoptosis such as BCL2, BCLXL, β 2-macroglobulin indicating roles in cell cycle and survival (326). This is confirmed as deletion of STAT-3 in T-cells leads to both impaired survival and impaired proliferation (327, 328).

STAT-3 has been heavily implicated in numerous cancers due to its persistent activation found to be present in numerous malignancies (329-331). This activation results in the proliferation, survival and immune evasion of tumours. In colorectal cancer the level of STAT-3 activation has been shown to correlate with tumour grade, lymph node metastasis and extent of tumour invasion (332). The over-activation of this transcription factor is associated with poorer prognosis of many cancers (333, 334). The ablation of STAT-3 gene expression in human and mouse tumour models results in the inhibition of tumour growth, highlighting the potential targeting of this molecule as an approach for effective cancer treatment (335-337). Numerous approaches have been studied in order to prevent STAT-3 activation including strategies to prevent the homo-dimerisation, nuclear translocation or DNA binding of STAT-3 (338-340). The inhibition of the tyrosine kinase pathway also represents an attractive therapeutic target by the indirect inhibition of STAT-3 and such molecules have been shown to cause tumour growth arrest and apoptosis (341). Challenges remain in the targeting of STAT-3 for cancer therapy; however, this does represent a promising therapeutic target in anti-tumour immunity.

The diverse functions of this transcription factor and its effect on many cell types highlights its pleiotropic nature emphasising the complex role of this transcription factor in immune responses.

1.3.8 IL-6 mediated STAT-3 Signalling

The activity of an IL-6 mediated STAT-3 signalling pathway in numerous autoimmune diseases marks it out as a potentially important common pathogenic component, and feature of disease pathogenesis, highlighted as the CD4⁺ T-cell specific deletion of STAT-3 results in resistance to experimental autoimmune uveoretinitis (EAU), experimental autoimmune encephalomyelitis (EAE) and autoimmune pneumonitis (342, 343).

The failure of autoimmune disease development with the deletion of STAT-3 in CD4⁺ T cells has been documented to be due to the role of the IL-6-STAT-3 signalling pathway, which has been shown to be an important regulator of the TGF- β driven differentiation of murine Th17 cells (342, 344-346). This pathway has also been shown to promote the differentiation of T-cells to Tfh cells with B-cell helper capacity (298, 347, 348). Despite the documented role of IL-6 in the inhibition of Treg development the presence of STAT-3 in these cells is crucial for their suppressive abilities and has been shown to protect against development of colitis (349).

In the oncology field, markers of STAT-3 mediated IL-6 signalling have been shown to be potential prognostic markers of breast cancer. Naïve CD4⁺ T-cells from peripheral blood of patients with breast cancer have significantly lower levels of phosphorylated STAT-1 (pSTAT-1) and pSTAT-3 showing reduced responsiveness to IL-6 (350). These responses were associated with defective Th17 differentiation and correlated with worse relapse free survival. In IBD, there is a high level of STAT-3 phosphorylation in both CD and ulcerative colitis (UC) patients (351). The level of pSTAT-3 has been shown to correlate with gut inflammation. It is thought that due to its role in mediating the proliferation and survival of CD4⁺ T-cells and Th17 differentiation this STAT-3 activation exacerbates colitis. Its role in the disease is confirmed as IL-6 deficient mice have reduced development of colitis (352). In the dextran sodium sulphate (DSS) induced colitis model STAT-3 phosphorylation is enhanced causing more severe colitis in F59J-JAB transgenic mice with a defect in SOCS-3, the negative regulator of STAT-3.

The importance of IL-6 mediated STAT-3 signalling in resistance to Treg suppression has been highlighted in MS and psoriasis where IL-6 is also found in high levels. The deletion of both IL-6 and STAT-3 lead to resistance against EAE (343). In patients with relapsing/remitting MS there is an elevated level of pSTAT-1:pSTAT-3 (353). This increased pSTAT-3 correlates with increased responsiveness to IL-6 and the resistance of cells to Tregs. Similarly, in psoriasis levels of IL-6 are elevated in both lesional skin and serum (354). This leads to the strong induction of pSTAT-3 which is prolonged in effector T-cells and promotes resistance of cells to Treg suppression. In both cases, this is thought to be a result of higher levels of IL-6R and/or gp130 on patient CD4⁺ T-cells.

1.3.9 Effect of IL-6 on CD4⁺ T-cells

In addition to its effects on B-cells and myeloid cells, IL-6 is increasingly understood to have profound effects on T-cell responses. As well as being responsible for the cytokine-mediated recruitment of CD4⁺ T-cells inflammatory site (355, 356), IL-6 regulates the activation, proliferation, apoptosis and differentiation of CD4⁺ T-cells, influencing the nature and duration of chronic inflammation. Its enhancement of PHA-induced T-cell proliferation occurs independently of IL-2 (357), whilst blockade of IL-6 with anti-IL-6R has been shown to suppress anti-CD3 anti-CD28 induced proliferation by the partial inhibition of IL-2 production (358). Similarly, IL-6 protects CD4⁺ T-cells from activation induced cell death (AICD) and rescues from apoptosis independently of IL-2 through the downregulation of FASL surface expression and the STAT-3 dependent upregulation of anti-apoptotic molecules such as BCL-2 (261, 359). IL-6 has been shown to play a role in the persistence of memory CD4⁺ T-cells: antigen-specific T-cells expanded more vigorously following immunisation with the addition of exogenous IL-6 as a result of decreased apoptosis (360).

Presence of IL-6 during CD4⁺ T-cell activation plays a crucial role in the differentiation of T-helper cell subsets with promotion of Th2, Th17 and Tfh cells and inhibition of Th1 and Treg cells being well documented. During the early stages of T-cell activation IL-6 has been shown to induce the expression of c-maf in naïve CD4⁺ T-cells. STAT-3 binds the c-maf promoter and subsequently regulates IL-4 transcription and commitment to the Th2 lineage (361). Furthermore, promotion of Th2 differentiation can be achieved by the presence of IL-6 during TCR stimulation, which has been linked to the production of IL-4 by CD4⁺ T-cells via the promotion of Nuclear factors of activated T-cells 2 (NFATc2) (362). The induction of c-maf alone is not sufficient for the development of Th2 cells suggesting induction of both pathways is necessary for Th2 differentiation. Alternatively, CD4⁺ T-cells activated in the presence of IL-6 have decreased Th1 differentiation due to the increased induction of SOCS1 resulting in inhibition of IFN- γ production (363). Together these data suggest IL-6 and TCR stimulation alters the balance of T-helper T-cells towards a Th2 response, indicating a possible role in allergy related diseases.

A number of studies have shown that culture of CD4⁺ T-cells with a combination of IL-6, TGF- β , IL-1 β and IL-23 results in the differentiation of pathogenic Th17 cells. The contributions of these various cytokines is controversial but it is important to note that many of these studies were carried out in mice and the contribution of IL-6 towards the skewing of human CD4⁺ T-cells towards Th17 is still debateable. In mice, IL-6 and TGF- β generate Th17 cells from a precursor also common to Foxp3 Tregs (364-366). These studies show variable roles for IL-23

and possible amplification by IL-1 β and TNF. In these mouse models, as IL-6R is proteolytically cleaved from the surface of activated CD4⁺ T-cells, development of Th17 cells is mediated by classical signalling whereas the maintenance of these cells is mediated by trans signalling (367, 368). In contrast, human Th17 cells have been shown to require IL-1 β and IL-23 or IL-1 β and IL-6 in their generation with no role for TGF- β (369, 370). IL-6 may play a role in human Th17 differentiation although further work is required to clarify the exact conditions required.

The trans signalling mechanism of IL-6 is well known to abrogate the induction of Foxp3 in naïve CD4⁺ T-cells through the induction of mothers against decapentaplegic homolog 7 (SMAD7), an inhibitor of the TGF- β signalling pathway (371). Despite the debate over the contribution of IL-6 to the development of Th17 the inhibition of STAT-3 in CD4⁺ T-cells prevented the differentiation of Th17 cells and increased the proportion of Tregs (372). This overall effect of IL-6 shifts the balance of T-helper cells away from Treg and immune tolerance.

Loss of immunological tolerance in autoimmune diseases may result from the failure of Tregs to control autoreactive effector responses (373). Numerous studies have suggested an apparent role for IL-6 in inhibiting Treg function, explained by its ability to render Teff cells resistant to Treg-mediated suppression via STAT-3 signalling (374, 375). A high pSTAT-3:pSTAT-1 ratio has recently been associated with the loss of Treg suppression (375). It has been shown that inhibition of pSTAT-3 restores Treg suppression, however, the presence of pSTAT-3 early in responses are required in both effector cells and Tregs in order for IL-6 to mediate the reversal of suppression. This mechanism of effector T-cell resistance to suppression has been demonstrated in mouse models of SLE, EAE and T1D (376-378). This pathway has also been highlighted as playing an important role in a number of human diseases, including SLE, T1D, JIA, MS and psoriasis (354, 379-383).

IL-6 has been shown to regulate the expression of BCL-6, the major transcription factor of Tfh cells, with a specific requirement of STAT-1 signalling (162, 384). One proposed mechanism identifies IL-6 mediated upregulation of BCL-3, a feature present in early RA, as a key driver of BCL-6 expression (164). IL-6 induces both the gene expression and protein induction of IL-21 in both naïve and memory CD4⁺ T-cells stimulated in the presence of IL-6 in a STAT-3 dependant manner (298, 385). Furthermore, deficiency of STAT-3 resulted in compromised expression of IL-21 and diminished B-cell helping capacity (348). IL-6 is not the only cytokine required for commitment to the Tfh cell lineage with many studies showing a role for IL-21 and more recently studies suggests roles for TGF- β and IL-12 or IL-23 (386, 387).

It has recently been shown that IL-6 can regulate effector function by its influence on mitochondrial membrane potential (388). This study shows that IL-6 in a STAT-3 dependent manner facilitates the formation of mitochondrial respiratory chain complexes enabling cells to sustain high mitochondrial membrane potential. This increased potential results in increased levels of mitochondrial Ca^{2+} , shown to prolong the expression of IL-4 and IL-21 late during T-cell activation. This reveals a novel mechanism by which IL-6 regulates effector cell functions.

This critical role for IL-6 in T-cell differentiation highlights its importance in the maintenance of effector responses and hence chronic inflammation and is summarised in *Figure 6*. The induction of pro-inflammatory Th17/Th2 and inhibition of Tregs indicates its importance in shifting the immune response towards chronic inflammation and disruption of immune tolerance.

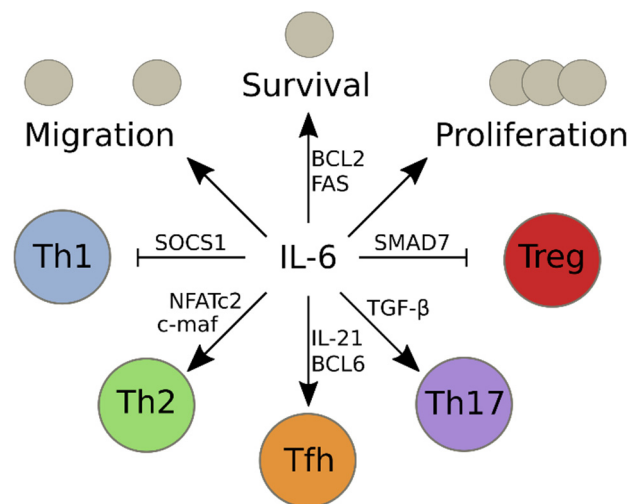


Figure 6. Effects of IL-6 on CD4⁺ T-cells. IL-6 has numerous effects on CD4⁺ T-cell function including the induction of cytokine mediated migration and increasing cell survival and proliferation. IL-6 also plays a role in CD4⁺ T-helper cell differentiation by promoting Th2, Tfh and Th17 cells and inhibiting development of Th1 cells and Tregs through the induction of certain transcription factors and cytokines.

1.3.10 Role of IL-6 in Inflammatory Arthritis

In RA there is a positive correlation between serum and SF IL-6 levels and markers of inflammation, such as acute phase proteins, disease activity and chronic synovitis (222, 389-392), with pre-treatment levels of IL-6 shown to predict bone erosion (393). In healthy individuals IL-6 levels are barely detectable within the range of 2-6pg/ml whereas in inflammatory diseases such as RA levels of up to 150ng/ml have been reported with only 2-5 fold increases in sIL-6R and sgp130 levels (291). This suggests that in disease states IL-6 levels exceed sIL-6R and sgp130 to act systemically.

Levels of sIL-6R are also elevated in RA, particularly in SF, which correlate with leukocyte infiltration (389, 390, 394, 395). Trans signalling promotes VEGF production by mesothelial cells which contributes to angiogenesis, inhibits proteoglycan synthesis in RA chondrocytes and promotes osteoclast-like cell formation all suggesting a role in cartilage and bone destruction (390, 396).

The role of IL-6 in RA has been further suggested by many mouse models. Mice deficient for IL-6 have been shown to be resistant to the induction of AIA or exhibit reduced disease severity, fewer osteoclasts and lower levels of IL-17 (397-399). One study has reported that IL-6 is essential for CIA development, however, other studies of IL-6 deficient mice have shown delayed onset and reduced severity of CIA with lower humoral and cellular responses (400, 401). Furthermore, in CIA the administration of anti-IL-6 blocking antibody reduces both the incidence and severity of disease, with reduced T-cell and B-cell proliferative response to mitogens (402). Finally, specific targeting of the trans signalling pathway with sgp130Fc has been shown to ameliorate experimental inflammatory arthritis highlighting the trans signalling pathway in disease pathogenesis (265).

Mouse models of arthritis have also been developed by altering the IL-6 signalling pathway. For example, mutations of the tyrosine residues which make up the phosphatase binding site of gp130 have been shown to induce inflammatory arthritis. These mutations cause enhanced STAT-3 activation resulting in CD4⁺ T-cell accumulation, autoantibody production and increased IL-17 which increases proliferation and decreases apoptosis (403). A further mouse model with a knock in mutation in gp130^{Y757F/Y757F} resulting in hyper-activation of STAT-3 although not directly resulting in arthritis causes increased severity of autoimmune arthritis which can be abolished by IL-6 targeted therapy (264).

In humans, an SNP in the gene ANKRD55, 150kb proximal to the IL-6 signal transducer gene (IL6ST), has been shown to have significant association with RA (404). The function of the ANKRD55 gene is unknown; however, IL6ST is a more compelling candidate as this plays a role in the IL-6 receptor complex, hence could alter IL-6 signalling. A polymorphism within the IL-6R gene has been shown to be associated with RA with the presence of alanine strongly correlates with circulating levels of the IL-6 and sIL-6R. This may impair classical IL-6R signalling by reducing IL-6R levels, which could be due to increased proteolytic cleavage of the membrane bound IL-6R (405, 406). Other risk variants within genes in the IL-6 signalling pathway have also been identified as being associated with RA, including those in TYK2 and STAT-3 genes (405, 407).

1.3.11 Therapeutic Targeting of IL-6 in RA

Due to its numerous roles in inflammation and its suggested role in RA pathogenesis, therapies that target the IL-6 signalling pathway have been extensively studied for use in the treatment of RA, Table 1.

It was first seen that treatment of RA with MTX decreases levels of IL-6 relating to better clinical outcome (408). Since the development of biologics a number of therapies targeting different aspects of the IL-6 signalling pathway have been investigated and now such a therapy has been approved for use in over 100 countries (409).

Tocilizumab (TCZ) is a humanised monoclonal antibody (mAb) which blocks the IL-6 binding site of the IL-6R. This antibody is effective at blocking both the classical and trans signalling pathways of IL-6. TCZ therapy has proved to be efficacious both alone and in combination with DMARDs with an efficacy similar to other approved biologics (410, 411). Importantly, this therapy has also been shown to be effective in early RA in patients not previously treated with DMARDs and in those refractory to treatment with anti-TNF and/or MTX (411, 412). Further investigations reveal the therapy is effective in reducing disease severity, delaying joint destruction and improving physical function. It has been suggested that the level of IL-6 in the SF of RA patients correlates with subsequent positive response to IL-6R inhibition (413).

Due to the relative success of anti-IL-6R in the treatment of RA the mechanism by which treatment results in disease improvement have also been studied. There have been documented improvements in bone resorption, cartilage turnover and decreases in chemokine levels with TCZ treatment (414, 415). Therapy has also been shown to reduce the expression of T-cell activation markers and chemokines and induce the expression of pro-repair genes (416). There has been controversy as to the effects of TCZ and its influence on CD4⁺ T-helper cell subsets. Some studies have suggested a decrease in Th17 cells with increased numbers of Tregs three month's post-TCZ treatment. Others suggest increased Tregs with no changes in Th17 numbers, or decreases in Th1, Th17 and Tregs with an increase in Th2 cells (417-420). This suggests the effects of the blockage of IL-6 signalling is highly complex and warrants further investigation.

Sarilumab, another mAb against IL-6R, has a similar efficacy as TCZ both in combination with MTX or as a monotherapy (421, 422). Further therapies are also being developed against the IL-6 ligand including Sirukumab and Olokizumab. A consequence of these drugs is that the level of circulating IL-6 does not increase following treatment, as seen with TCZ, the consequences of this remain unclear, however, phase II clinical trials of these molecules so far suggests reduced symptoms of RA in those unresponsive to anti-TFN or MTX (423-425).

Clinical trials are now underway to specifically block the trans signalling pathway of IL-6 using the sgp130Fc, Olamkicept. Phase I trials have proved the safety of this drug which will progress to Phase II trials (426). Treatments which specifically target the trans signalling pathway of IL-6 hold much promise in the treatment of numerous inflammatory diseases due to the various pathogenic consequences of this signalling pathway described in *Chapter 1.2*.

Although it is not clear how the blockade of IL-6 results in improvement of RA, the clinical efficacy of these treatments highlights the importance of IL-6 in RA pathogenesis.

Mode of action	Therapy	Stage of development
Binds IL-6R	Tocilizumab	Approved for use
	Sarilumab	Approved for use
Binds IL-6	Sirukumab	Phase III trials
	Olokizumab	Phase II trials
Binds IL-6/sIL-6R	Olamkicept	Phase I/II trials
Binds JAK molecules	Tofacitinib	Phase III trials

Table 1. IL-6 directed therapies for the treatment of RA. Numerous molecules, which target the IL-6 signalling pathway, have been approved for use or are in clinical development for use in the treatment of RA. These drugs target different parts of the IL-6 pathway such as binding of IL-6R, sIL-6R, IL-6 or JAK molecules. Sirukumab is no longer in development.

1.3.12 IL-6 mediated STAT-3 Signalling in RA

Work carried out on the STAT-3:STAT-1 balance in CD4⁺ T-cells as a predictive biomarker for early RA development suggests that IL-6 mediated STAT-3 signalling in resistance to Treg suppression may also act in this disease setting (427). Teff resistance may be relevant in the pathogenesis of RA with studies showing that cytokines such as TNF- α , IL-2 and IL-7 are able to abrogate Treg suppression in established chronic RA (157, 158). Treatment of RA with TCZ is associated with an increase in Treg numbers and decreases in Th17 cells and a further study has shown TCZ treatment results in improved levels of suppression further confirming the possibility of this mechanism in disease pathogenesis (428-430).

As previously mentioned in *Chapter 1.3.10*, numerous experimental arthritis mouse models implicate IL-6 signalling in their pathogenesis. Mechanisms have been further elucidated showing the effects of IL-6 are mediated by STAT-3 signalling thus highlighting the role of this pathway in the pathogenesis of RA. For example, the deletion of STAT3 in gp130^{Y757F} mice resulted in reduced arthritis severity and reduced synovial infiltration suggesting a key role of this pathway in regulating the inflammatory infiltrate (264).

The global gene expression of circulating CD4⁺ T-cells of early RA patients have been profiled by Pratt et al. with the aim of obtaining biomarkers that predict the development of RA whilst shedding light on pathogenesis (431). This study identified the robust differential expression of 12 genes between untreated early RA and non-RA patients. This “12-gene signature” provided added value to existing tools for predicting the progression to RA amongst patients with undifferentiated arthritis. It did this most reliably in the most diagnostically challenging subgroup of patients, the seronegative RA subgroup.

Pathway analysis revealed that the 12-gene signature comprised a statistically significant over representation of genes regulated by STAT-3. These genes were found to be maximally upregulated in seronegative disease and include B-cell lymphoma-3 (BCL3), PIM1, SOCS3, strawberry notch homolog 2 (SBNO2) and mucin 1 (MUC1). The expression of these genes was found to correlate with paired serum IL-6 levels but not with any of the other relevant serum mediators investigated including TNF, IL-1 α , IL-10, leptin, IL-17 and IL-23. More recently, findings in relation to BCL3, PIM1 and SOCS3 have been validated in an independently recruited early arthritis cohort of 161 patients. Meta-analysis of the data from the two cohorts has confirmed the atypical IKB-encoding BCL3 as the single most differentially expressed gene, independent of baseline clinical parameters including measures of systemic inflammation (*Figure 7, Table 2*; Pratt et al, manuscript under review).

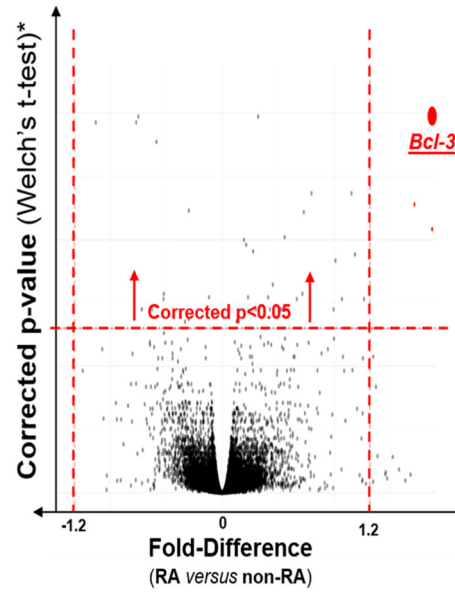


Figure 7. Volcano plot representing global CD4⁺ T-cell gene expression amongst early arthritis patients. Bcl-3 has the highest fold difference between 334 RA patients and non-RA (3.4 fold), which is highly significant following Benjamini-Hochberg multiple test correction. Figure from Pratt et al, manuscript in preparation.

	B	SE	Wald	df	p-value	Exp[B] (95% CI)
BCL3	.002	.001	7.031	1	0.008	1.002 (1.001-1.004)
Age	.035	.014	6.694	1	0.010	1.036 (1.009-1.064)
CRP	-.025	.016	2.516	1	0.113	0.975 (0.945-1.006)
ESR	.006	.011	.317	1	0.573	1.006 (0.985-1.027)
SJC28	.169	.083	4.131	1	0.042	1.184 (1.006-1.394)
Constant	-4.392	.970	20.492	1	0.000	.012

Table 2. Logistic regression in respect of RA versus non-RA diagnoses, with BCL-3 an independent variable alongside age, CRP, ESR and swollen joint count (SJC). Figure from Pratt et al, manuscript in preparation.

Additional studies have highlighted a strong correlation between constitutive intracellular pSTAT-3 and serum IL-6 that is unique to CD4⁺ T-cells and not seen in other circulating leukocyte subsets of early arthritis patients (427). This relationship appears to be the strongest in the true naïve CD4⁺ T-cell subset (CD45RA⁺CD62L⁺; *Figure 8A*, unpublished data). This suggests that, compared with central memory and effector memory CD4⁺ T-cells, the naïve CD4⁺ T-cell population is maximally sensitive to circulating IL-6. To examine whether this observation related to a higher propensity for IL-6 classical signalling in naïve cells due to higher availability of membrane bound IL-6R, its expression was examined on different subsets

of CD4⁺ T-cells. IL-6R expression was in fact expressed maximally on central memory CD4⁺ T-cells opposed to naïve CD4⁺ T-cells, which have maximal pSTAT-3 (*Figures 8B and C*, unpublished data). This data suggests naïve CD4⁺ T-cells are most sensitive to IL-6 through a mechanism not fully explained by levels of IL-6R expression.

The importance of the IL-6-STAT-3 pathway in early RA is corroborated by further studies showing baseline pSTAT-3 is found in 56% of RA patient CD4⁺ T-cells and levels correlated with DAS28 and treatment response (432). Levels of pSTAT-3 have also been shown to be higher in early RA than healthy controls, which correlated with elevated levels of IL-6 (433).

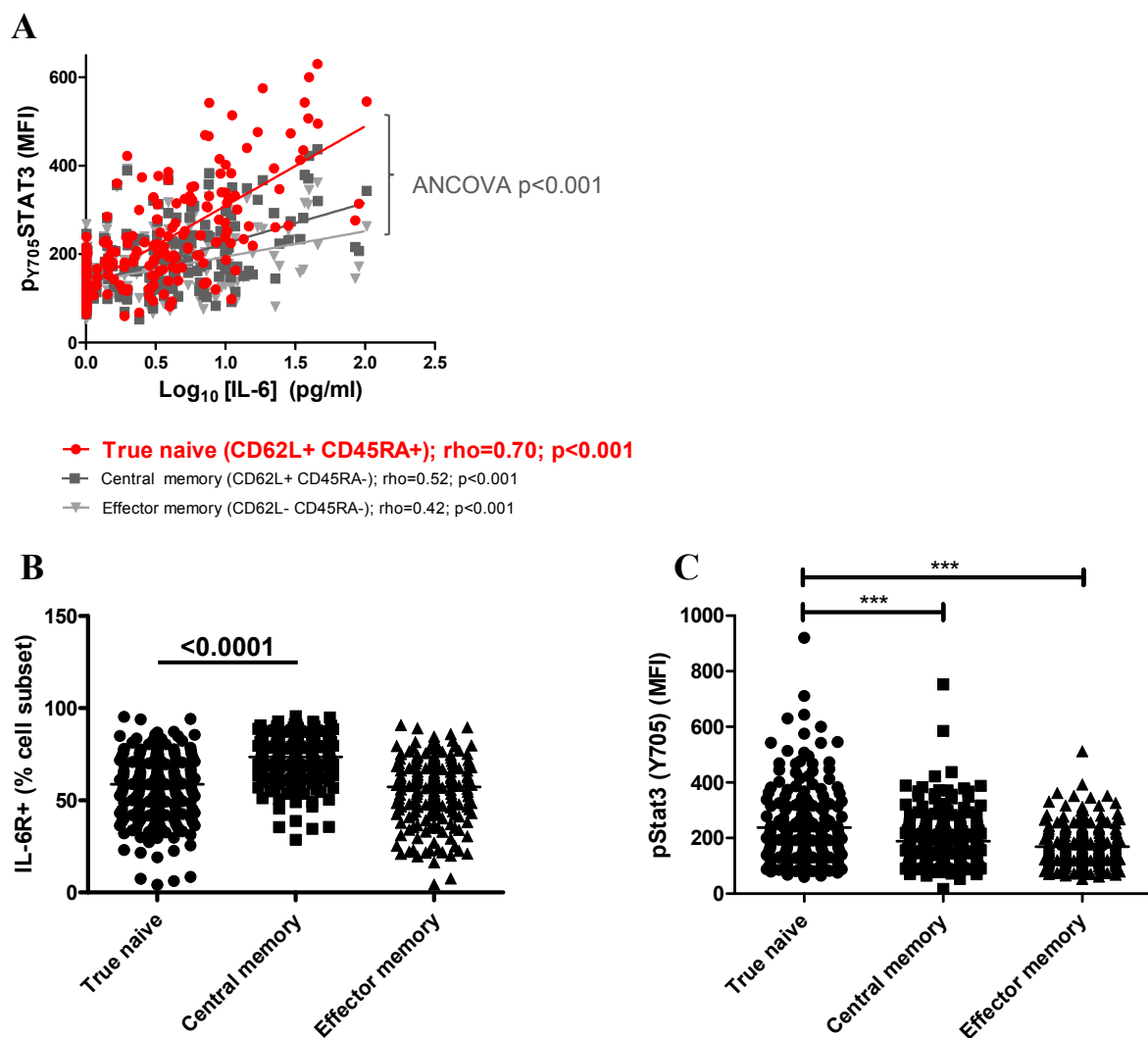


Figure 8. Naïve CD4⁺ T-cells are maximally sensitive to paired serum IL-6 by a mechanism other than IL-6R expression. (A) There is a strong correlation between constitutive STAT-3 phosphorylation and paired serum IL-6 in CD4⁺ T-cells; with the relationship strongest in true naïve CD4⁺ T-cells. Significant differences occur between best fit line slopes (analyses of covariance p<0.001) (B) IL-6R expression is highest on central memory CD4⁺ T-cells and lower on effector memory and true naïve CD4⁺ T-cells. (C) STAT-3 phosphorylation is highest in true naïve CD4⁺ T-cells compared to central memory and effector memory, significance determined by non-parametric analysis of variance (Friedman) with Dunn's posthoc pairwise analyses, ***p<0.001.

1.4 Hypothesis

The observational data generated in early RA showing high level of circulating IL-6 which correlate with significantly enhanced STAT-3 gene expression highlights IL-6 mediated STAT-3 signalling as an early event in RA pathogenesis. This raises important questions as to the mechanisms via which IL-6 induced gene induction in CD4⁺ T-cells of early RA patients may play a role in the induction of immune dysregulation. I hypothesise that in early RA circulating naïve CD4⁺ T-cells are chronically pre-exposed to IL-6, which mediates STAT-3 activation and subsequent aberrant effector function following TCR stimulation providing a mechanism of antigen non-specific immune dysfunction in early RA.

Therefore, the aims of this thesis are:

1. To establish a robust in vitro model to mimic chronic IL-6 pre-exposure of naïve and memory CD4⁺ T-cells and subsequent TCR-mediated activation.
2. To determine the phenotypic consequences of chronic IL-6 pre-exposure on naïve and memory CD4⁺ T-cell effector function following TCR-mediated stimulation.
3. To investigate the molecular consequences of IL-6 pre-exposure by identifying transcripts differentially induced in naïve and memory CD4⁺ T-cells as a result of IL-6 pre-exposure.
4. To correlate findings from the effects of IL-6 pre-exposure to the situation in primary CD4⁺ T-cells from early RA patients.

Chapter 2. Materials and Methods

2. 1. Reagents

RF10 – Roswell Park Memorial Institute media 1640 (RPMI 1640; Sigma Aldrich, UK) supplemented with 5% fetal calf serum (FCS; Thermo Fisher Scientific; Massachusetts, USA), penicillin (50U/ml; Sigma Aldrich), streptomycin (50mg/ml; Sigma Aldrich) and glutamine (2mM; Sigma Aldrich)

TexMACS – (Miltenyi Biotech; Cologne, Germany) supplemented with 100U/ml penicillin and 100µg/ml streptomycin

IMDM + 10% FCS – Iscove's Modified Dulbecco's Medium (IMDM; Thermo Fisher Scientific) supplemented with 10% FCS, 2mM glutamine, 100U/ml penicillin and 100µg/ml streptomycin

IMDM + 10% SR – IMDM supplemented with 10% serum replacement (SR; Invitrogen; California, USA), 2mM glutamine, 100U/ml penicillin and 100µg/ml streptomycin

Xvivo – Xvivo-15 (Lonza) supplemented with 100U/ml penicillin and 100µg/ml streptomycin

HBSS + EDTA - Hanks Balanced Salt Solution; Ca^{2+} and Mg^{2+} free (HBSS; Lonza; Basel, Switzerland) supplemented with 2mM ethylene diamine tetra-acetic acid (EDTA; Thermo Fisher Scientific)

HBSS + 1% FCS – HBSS; Ca^{2+} and Mg^{2+} free (Lonza) supplemented with 5ml FCS (Labtech; Sussex, UK)

PBS + 2% FCS – Phosphate buffered saline; Ca^{2+} and Mg^{2+} free (PBS; Lonza) supplemented with 10ml FCS (Labtech)

Lymphoprep – (Axis-Shield Diagnostics; Dundee, UK)

FACS buffer – PBS (Lonza) containing 0.5% bovine serum albumin (BSA; Sigma Aldrich), 1mM endotoxin free EDTA and 0.01% sodium azide (Sigma Aldrich)

MACS buffer – PBS (Lonza) containing 0.5% FCS and 0.5M EDTA

Binding buffer – 0.1M Hepes (pH 7.4; Sigma Aldrich) with 1.4M NaCl (Sigma Aldrich) and 2.5mM CaCl_2 (Sigma Aldrich)

ELISA wash buffer – PBS (0.14M NaCl, 8.52mM Na_2HPO_4 , 1.59mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (BDH Chemicals)) with 0.1% Tween 20 (Sigma Aldrich)

ELISA block – PBS (0.14M NaCl, 8.52mM Na₂HPO₄, 1.59mM NaH₂PO₄.H₂O) containing 1% BSA

ELISA diluent – PBS (0.14M NaCl, 8.52mM Na₂HPO₄, 1.59mM NaH₂PO₄.H₂O) containing 1% BSA and 0.1% Tween 20

Citrate Phosphate Buffer – 0.03M citric acid (Sigma Aldrich), 0.05M Na₂HPO₄, 0.03M Na₂HPO₄.2H₂O and H₂O (6μl, 30% stock)

3M Sulphuric acid – 3M H₂SO₄ (Sigma Aldrich) diluted in 250ml dH₂O

4% formaldehyde – 100ml PBS (Lonza) containing 4g paraformaldehyde (Sigma Aldrich)

2.2. Subjects

The subjects used for the study mainly comprised of healthy volunteers and patients recruited from the Northeast Early Arthritis Cohort (NEAC). All donors gave written informed consent for inclusion in the study prior to giving blood. Ethical approval for patient samples was obtained from Newcastle and North Tyneside 2 Research Ethics Committee (reference 12/NE/0251). Ethical approval for healthy control blood was obtained from Newcastle Academic Health Partners Bioresource and Newcastle and North Tyneside 1 Research ethics Committee (reference 12/NE/0395, R&D 6579).

2.2.1. National Blood Service

Leukocyte reduction system (LRS) cones from healthy donors were provided by the National Blood Service. This is a way of eliminating leukocytes from blood products such as platelets. Cones provide a viable source of white blood cells which can be used for further cell isolations.

2.2.2 Healthy Laboratory Donors

Peripheral blood was obtained from healthy laboratory donors from the Musculoskeletal Research Group. According to an established standard operating procedure, healthy donors volunteered in response to an e-mail sent to people on the blood donation list and provided fully informed consent prior to blood donation. Ethical approval was obtained from Newcastle and North Tyneside 1 research ethics Committee (reference 12/NE/0395, R&D 6579).

2.2.3 Early Arthritis Patients

Patients were recruited from the Musculoskeletal Unit at the Freeman Hospital, Newcastle upon Tyne. Having provided fully informed consent for enrolment into the Northeast Early Arthritis Cohort (NEAC; REC 12/NE/0251), volunteers donated blood prior to commencement of immune-modulatory treatments (including steroids and DMARDs). Diagnoses and

clinical/laboratory parameters were recorded on a secure database at the time of blood draw, using codes to identify patients, and diagnoses could be confirmed at follow-up.

2.3 Obtaining Peripheral Blood

A volume of blood (between 70-140ml) was drawn into ethylene diamine tetra acetic acid (EDTA) or citrate vacuette tubes (Greiner Bio-One; Kremsmünster, Austria).

Numerous studies have shown differences in cell properties as a result of anti-coagulant use, possibly due to the metal cation chelation property of these substances altering the availability of Ca^{2+} involved in metabolic processes. For example, studies describe higher cell yield with blood drawn into EDTA although other studies show lower levels of cytokine secretion and protein secretion of monocytes and impaired CD4^+ T-cell proliferation with this anti-coagulant (434-439). In contrast, citrate was reported to result in high quality RNA/DNA and smaller variation in gene expression which may be due to the lower binding affinity of this anti-coagulant (440, 441).

Due to this the effects of EDTA and citrate on naïve CD4^+ T-cell proliferation was compared. This revealed that naïve CD4^+ T-cells from blood stored in citrate were more sensitive to changes in proliferation, data not shown. For this reason, citrate was chosen as the preferred anti-coagulant.

In some cases sera was also collected into serum/gel vacuette tubes (Greiner Bio-One).

2.4 Cell Isolation

A variety of cell isolation methods were used in this study to obtain both total CD4^+ T-cells and naïve CD4^+ T-cells. A number of different naïve CD4^+ T-cell isolation methods were compared in order to choose the method of isolation which gave both a high yield and high purity. Purity of all isolated cells was assessed by subjecting aliquots of cells to flow cytometry.

2.4.1 Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) can be isolated from fresh whole blood using density centrifugation on density medium. On centrifugation cells separate according to density with heavier cells including red blood cells (RBCs) and granulocytes forming a pellet at the bottom of the tube. PBMCs (including lymphocytes and monocytes) form a layer at the interface which can be recovered for further use.

Fresh whole blood or LRS cones was diluted 1:2 or 1:3 respectively with HBSS + EDTA (*Chapter 2.1*). 15-20ml of diluted blood was then slowly layered over 15ml Lymphoprep (*Chapter 2.1*) and centrifuged at 895g for 30 minutes at room temperature. PBMCs were

recovered from the plasma interface with a sterile Pasteur pipette and transferred to a new 50ml Falcon tube. Cells were resuspended in 50ml HBSS + 1% FCS (*Chapter 2.1*) and centrifuged at 600g at 4°C for 7 minutes to remove any contaminating Lymphoprep. Supernatant was discarded and cells washed again by resuspending in HBSS + 1% FCS and centrifuging at 250g at 4°C for 7 minutes. The supernatant was then aspirated which will remove any remaining platelets. At this stage, PBMCs were re-suspended in an appropriate amount of RF10 (*Chapter 2.1*), counted using a Burkert chamber, and then used for further downstream applications.

2.4.2 Isolation of total CD4⁺ T-cells by positive selection

Monocytes express low levels of CD4 so must be depleted from blood using CD36 RosetteSep before positive CD4⁺ T-cell isolation. This crosslinks CD36 on the surface of monocytes with RBCs to form heavy density immunorosettes. On addition of an erythrocyte aggregation agent, these rosettes then pellet and leukocytes can be recovered for further use. CD4⁺ T-cells can then be isolated through the binding of tetrameric antibody complexes to CD4 which are then bound by dextran coated magnetic particles. These cells can then be separated using magnetic field.

Fresh whole blood was incubated with monocyte depleting CD36 RosetteSep (25µl/ml; Stemcell Technologies; Vancouver, Canada) at room temperature for 20 minutes. Hetasep (Stemcell Technologies) was added, at 1/5 of the starting volume of blood, and centrifuged at 50g room temperature for 5 minutes. Cells were left to stand for 10 minutes and supernatant containing desired leukocytes transferred to a new universal. Cells were then centrifuged at 400g for 7 minutes at room temperature. Cells were resuspended in a volume of HBSS + 1% FCS equivalent to 1/5 the starting volume of blood and loaded along with other reagents onto the Robosep automated cell separator (Stemcell Technologies) which was programmed for human CD4 whole blood positive selection and run according to manufacturer's protocol. Briefly, this involves incubation of sample with CD4 antibody complexes for 15 minutes followed by incubation with magnetic particles for further 10 minutes which cross link with the CD4 antibody. Cells were incubated in the magnetic field of an EasySep magnet for 5 minutes following which cells unbound to magnetic particles are removed by pipetting off supernatant. Positively selected CD4⁺ T-cells were manually removed from the robosep magnet and resuspended in culture media for cell counting.

2.4.3 Isolation of total CD4⁺ T-cells by enrichment

CD4⁺ T-cells were enriched from the blood by density centrifugation. The human RosetteSep CD4⁺ T-cell enrichment cocktail (Stemcell Technologies) forms immunorosettes by cross-linking antibodies on all CD4 negative cells to RBCs. On centrifugation over density medium, these form a pellet and unlabelled CD4⁺ T-cells can be recovered from the interface.

Total CD4⁺ T-cells were isolated from fresh whole blood or LRS cones by incubating with Human CD4⁺ T-cell Enrichment Cocktail as per manufacturers instructions (50µl/ml for blood or 75µl/ml for cone, Stemcell Technologies) for 20 minutes at room temperature. Samples were then diluted 1:2 or 1:3 respectively with PBS+ 2% FCS (*Chapter 2.1*) and 15-20ml was layered over 15ml Lymphoprep. Cells were then separated by centrifugation at 895g, room temperature for 30 minutes. CD4⁺ T-cells were recovered from the interface using a sterile pasteur pipette and were centrifuged at 600g at room temperature for 7 minutes to remove contaminating Lymphoprep. Another 400g for 7 minutes room temperature centrifuge was carried out and cells were then resuspended in culture media for counting.

2.4.4 Isolation of naïve CD4⁺ T-cells by EasySep enrichment

Naïve CD4⁺ T-cells can be enriched from blood by the use of tetrameric antibody complexes recognising surface antigens of all CD45RA negative cells. These antibodies bind to their target cells and are subsequently labelled with dextran coated magnetic nanoparticles. These cells are then separated from unlabelled naïve CD4⁺ T-cells using the magnetic field of an EasySep magnet (Stemcell Technologies).

PBMCs were isolated from fresh whole blood or LRS cones by gradient density centrifugation as previously described (*Chapter 2.4.1*). Naïve CD4⁺ T-cells were purified from PBMCs at a concentration of 5x10⁷ cells/ml by incubating with biotinylated anti-CD45RO antibody (50µl/ml; Stemcell Technologies) for 15 minutes at room temperature. Cells were incubated with EasySep Human Naïve CD4⁺ T-cell Enrichment Cocktail (50µl/ml; Stemcell Technologies) for 10 minutes at room temperature. Nanoparticles were then added (100µl/ml; Stemcell Technologies) and incubated at room temperature for 10 minutes. The cell suspension was then made up to a total of 2.5ml and passed through a magnet for 10 minutes. The desired fraction was poured off into a new tube. This process was repeated for two separations with the magnet in order to improve purity. This left the negatively selected naïve CD45RA⁺ T-cells which were re-suspended in culture media for counting.

2.4.5 Isolation of naïve CD4⁺ T-cells using PluriBeads

Naïve CD4⁺ T-cells can be positively selected by non-magnetic mono-dispersed micro-particles with mAbs on the surface which recognise CD45RA on naïve CD4⁺ T-cells. Cells are isolated by filtration with the naïve CD4⁺ T-cells bound to the pluriBeads (PluriSelect Life Science; Leipzig, Germany) being unable to pass through the cell strainer. Target cells are then detached from the beads by addition of detachment buffer and naïve cells collected.

Naïve CD4⁺ T-cells were isolated from fresh whole blood by adding stabilisation buffer (50µl/ml; PluriSelect Life Science) followed by pluriBeads (80µl/ml; PluriSelect Life Science) and incubating for 30 minutes. Cells were then strained through S-pluriStrainer (PluriSelect Life Science) into 50ml centrifuge tube. Unlabelled cells run through the strainer whereas desired naïve CD4⁺ T-cells with rosette beads remain on the strainer. The strainer was washed with 5x 2ml wash buffer (PluriSelect Life Science) and attached to a new 50 ml centrifuge tube. It was then washed with 1ml wash buffer followed by 1ml detachment buffer and incubated for 10 minutes at room temperature. The strainer was washed with a further 1ml wash buffer (PluriSelect Life Science). The Luer lock was then opened allowing the isolated cells to pass through. The strainer was washed with 10x 1ml wash buffer (PluriSelect Life Science). Cells were then centrifuged at 400g for 7 minutes and supernatant aspirated. Cells were then re-suspended in an appropriate amount of media for counting.

2.4.6 Isolation of naïve CD4⁺ T-cells by MACS enrichment

In this method naïve CD4⁺ T-cells are isolated from a starting population of PBMCs by magnetic-activated cell sorting (MACS). Undesired cells are labelled with biotin-conjugated mAbs recognising cell surface antigens of CD45RA negative cells. These are then magnetically labelled with microbeads conjugated to monoclonal anti-CD61 and anti-biotin antibodies. Labelled cells are depleted when they are retained on a MACS separation column in the magnetic field of a MACS separator (Miltenyi Biotec). Unlabelled naïve CD4⁺ T-cells are collected as they pass through the column.

PBMCs were isolated from fresh blood as previously described (*Chapter 2.4.1*). PBMCs were re-suspended in MACS buffer (40µl per 10⁷ total cells; *Chapter 2.1*) and incubated with biotinylated antibody cocktail (10µl per 10⁷ cells) for 5 minutes, 4°C. MACS buffer (30µl per 10⁷ total cells) and Naïve CD4⁺ T-cell MicroBead Cocktail II (20µl per 10⁷ total cells; Miltenyi Biotec) were added before incubation for 10 minutes, 4°C. MACS buffer was added to a total volume of 500µl. Cells were then applied to LS column (Miltenyi Biotec) in the magnetic field of a MACS separator. The column was washed with 3x 3ml MACS buffer. The negative fraction, the naïve CD4⁺ T-cells, flow through the column and were collected. Cells were re-suspended in culture media for counting.

2.4.7 Isolation of naïve CD4⁺ T-cells by CD45RA/RO microbeads

Naïve CD4⁺ T-cells are isolated from a starting population of enriched CD4⁺ T-cells, as previously described in *Chapter 2.4.3*, by addition of magnetically labelled CD45RA or CD45RO MicroBeads (Miltenyi Biotec). The cell suspension is passed through a MACS separation column in the magnetic field of a MACS separator. In the case of CD45RA

microbeads naïve CD4⁺ T-cells are magnetically labelled and retained in the column, these can be removed from the magnetic field and eluted. In the case of CD45RO microbeads unlabelled naïve CD4⁺ T-cells are collected as they pass through the column.

Naïve CD4⁺ T-cells were isolated from purified CD4⁺ T-cells by re-suspending cells in MACS buffer (80µl/10x10⁶ cells) and incubating with CD45RA/RO MicroBeads (Miltenyi Biotec) for 15 minutes at 4°C. Cells were then washed in 2ml MACS buffer and centrifuged at 700g for 7 minutes. Cells were resuspended in 50µl MACS buffer and applied to an LS column in the magnetic field of a MACS separator. The MACS column was washed with 3x 3ml MACS buffer. The negative fraction of naïve CD4⁺ T-cells flow through the column and were collected. Cells were then re-suspended in culture media for counting.

2.5 Cell Counting

Cell counting was carried out by diluting cells 1:1 with media and/or 0.4% trypan blue (Sigma Aldrich). Trypan blue is able to identify dead cells by passing through the membrane and making cells appear blue. Cells were then loaded onto the cover slip of a Burker chamber, Haemocytometer (Marienfeld Superior; Germany) and the number of cells in 25 squares was counted, including those touching upper and right borders, using a M20 phase contrast microscope (Wild; Switzerland) at 40X objective. The number counted was multiplied by the dilution factor to give cells x10⁴/ml. All subsequent cell counts were carried out in this way.

2.6 Cryopreservation of CD4⁺ T-cells

In some cases, cells were frozen following isolation before use in culture. Cryopreservation allows the cells to be stored for a long length of time before use. Cells can be preserved in dimethyl sulfoxide (DMSO) which protects biological material from freezing damage due to ice formation.

To do this, cells were centrifuged 400g for 7 minutes and re-suspended in 1ml of FCS + 10% DMSO (Sigma Aldrich) per 10x10⁶ cells. Cells were transferred to cryovials (Corning; New York, USA) and gradually cooled to -80°C in polystyrene box.

Upon thawing cells were placed in water bath until defrosted. Cells were then washed twice at 400g for 7 minutes re-suspended in culture media and counted for further use.

2.7 In Vitro Cell Culture

Following isolation total CD4⁺ T-cells, naïve CD4⁺ T-cells or memory CD4⁺ T-cells were cultured at 37°C with 5% CO₂ in 24 well or 96 well flat bottom culture plates (Costar, Corning)

unless stated otherwise. Cells were examined under a CKX41 inverted microscope (Olympus Corporation; Tokyo, Japan) at appropriate times throughout culture.

Cells were then used in subsequent flow cytometry phenotype or proliferation assays, *Chapter 2.9*. Supernatants from these cultures was stored at -20°C for subsequent cytokine measurement by ELISA or MSD, *Chapter 2.10*.

2.7.1 IL-6 pre-exposure

Purified naïve CD4⁺ T-cell and memory CD4⁺ T-cells were cultured at 1x10⁶ in 500µl TexMACs (Miltenyi Biotec) or Xvivo-15 (Lonza) in 24 well culture plates (Costar, Corning). A concentration titration was carried out using recombinant human IL-6 (20.9kDa; Peprotech; London, UK) at final concentrations of 50ng/ml, 5ng/ml, 0.5ng/ml and 0.1ng/ml with respective equimolar final concentrations of sIL-6R (37.6kDa; Peprotech). Stocks of IL-6 and equimolar sIL-6R were made by diluting with TexMACs or Xvivo-15 media then mixing with cells at a 1:1 ratio. Cells were cultured for 3 days unless stated otherwise, then harvested and centrifuged twice at 400g for 7 minutes to wash away IL-6. Cells were then stimulated by anti-CD3 and anti-CD28 (*Chapter 2.7.3*), in a mixed lymphocyte reaction (*Chapter 2.7.4*) or differentiated into T-helper subsets (*Chapter 2.7.5*).

2.7.2 Cytokine stimulation

Total CD4⁺ T-cells were isolated by positive selection using the robosep, *Chapter 2.4.2*. Cells were cultured in 24 well plates at 1x10⁶ cells in 500µl TexMACs alone or in 500µl TexMACs with final concentrations of 50ng/ml IL-6, TNF-α (17.4kDa; Peprotech), IL-21 (15.5kDa; Miltenyi Biotec), IL-23 (53.5kDa; Peprotech), IL-27 (47.8kDa; Peprotech) or IFN-α (gift from Catharien Hilkens, Newcastle University) for 1, 6 and 24 hours at 37°C with 5% CO₂.

2.7.3 Anti-CD3 and anti-CD28 stimulation

T-cells can be stimulated by anti-CD3 and anti-CD28, which mimic the stimulus from an antigen-presenting cell. Anti-CD3 provides the initial activation signal and a co-stimulatory signal is provided by anti-CD28 resulting in proliferation. This method of CD4⁺ T-cell activation provides the ability to accurately control strength of stimulus which is advantageous when measuring outcome following stimulation.

Anti-CD3 (OKT3; eBioscience; San Diego, USA) was immobilised on to 96 well flat bottom culture plate (Costar, Corning) at a final concentration of 1µg/ml (high), 0.5µg/ml (medium) or 0.2µg/ml (low). 2x10⁵ naïve CD4⁺ T-cells were added to each well in 150µl TexMACs or Xvivo-15 medium and soluble anti-CD28 (CD28.2; Biolegend; San Diego, USA) was added at

final concentration of 5µg/ml (high) or 1µg/ml (medium and low) in a final volume of 200µl, unless otherwise stated.

All culture incubations following anti-CD3 anti-CD28 stimulation were carried out for 6 days at 37°C with 5% CO₂ unless stated otherwise.

For intracellular staining cells were harvested at day 6, centrifuged 400g 7 minutes and resuspended in TexMACs or Xvivo-15 at a concentration of 1x10⁶ cells/ml. Cells plated out to 24 well plates and rested overnight at 37°C with 5% CO₂.

2.7.4 Mixed lymphocyte reactions

A mixed lymphocyte reaction (MLR) occurs when T-cells from one donor are co-cultured with allogeneic DCs from a second donor. T-cells will recognise the DCs as foreign due to the different MHC molecules resulting in T-cell activation and proliferation. This method of CD4⁺ T-cell activation is less accurately controlled, compared to anti-CD3 anti-CD28 stimulation, although is more physiologically relevant.

1x10⁵ naïve CD4⁺ T-cells were co-cultured with 1x10⁴ mature DCs. Mature DCs were isolated using CD14 MicroBeads (Miltenyi Biotech) and cultured in the presence of IL-4 and GM-CSF both at 50ng/ml for 6 days followed by culture with 0.1µg/ml lipopolysaccharide (LPS) for 24 hours to generate mature DC (from Katie Crossland, Newcastle University). Cells were cultured at a 1:10 ratio in a total volume of 200µl TexMACs (Miltenyi) or RF10 medium in triplicate in a flat bottom 96 well plate (Costar, Corning) for 6 days at 37°C with 5% CO₂. After 6 days, cells were used for tritiated thymidine incorporation assays (*Chapter 2.8.2*).

2.7.5 T-helper cell differentiation

Naïve, memory or total CD4⁺ T-cells were cultured at 1x10⁶ cells per well of a 24 well plate (Costar, Corning). For Th1 and Th2 skewing, naïve CD4⁺ T-cells were cultured in IMDM + 10% FCS (*Chapter 2.1*); however, for differentiation into Th17 cells, memory CD4⁺ T-cells were cultured in IMDM + 10% SR (*Chapter 2.1*). For Tfh cell differentiation, total CD4⁺ T-cells were cultured in TexMACs or IMDM + 10% FCS (*Chapter 2.1*).

The conditions for the generation of T-helper cell subtypes can be seen in *Table 3*. For the generation of Th1 cells 10 IU/ml IL-2 (15kDa; Roche; West Sussex, UK), 1ng/ml IL-12 (75.0kDa; Peprotech), 10µg/ml anti-IL-4 (Biolegend) were added and anti-CD3/anti-CD28 T-cell expander Dynabeads (Invitrogen) were added at a ratio of 1 bead per 10 cells.

For the generation of Th2 cells 10 IU/ml IL-2 (Roche), 20ng/ml IL-4 (15.1kDa; Miltenyi Biotech), 10µg/ml anti-IL-12 (Biolegend) and 10µg/ml anti-IFN-γ (eBiosciences) were added

and anti-CD3/anti-CD28 T-cell expander Dynabeads (Invitrogen) were added at a ratio of 1 bead per 10 cells.

For the generation of Th17 cells 10ng/ml IL-1 β (17.3kDa; Peprotech), IL-23 (Peprotech) and TGF- β (25.0kDa; Peprotech) was added and anti-CD3/anti-CD28 T-cell expander Dynabeads (Invitrogen) were used at a ratio of 1 bead per 50 cells.

For the generation of Tfh cells 10ng/ml IL-12 (Peprotech), 10ng/ml IL-6 (Peprotech), 10ng/ml IL-1 β (Peprotech) and 10ng/ml TGF- β (Peprotech) was added to each well. Anti-CD3/anti-CD28 T-cell expander Dynabeads (Invitrogen) were added at a ratio of 1 bead per 10 cells.

Cells were cultured for 6 days at 37°C with 5% CO₂ and either split or refreshed as necessary, which was indicated by media colour and/or cell clumping.

On day 6 of culture cells were harvested into EasySep tubes (Stemcell Technologies) and placed in EasySep magnet (Stemcell Technologies) for 5 minutes to remove anti-CD3/anti-CD28 T-cell expander Dynabeads (Invitrogen). The magnetic beads are attracted to the magnet and the CD4⁺ T-cells were poured off into new universal topped with HBSS + 1% FCS and centrifuged

T-helper skewing	Cell type	CD3/CD28 bead:cell ratio	Cytokines	Refresh/Split
Th1	Naïve CD4 ⁺ T-cells	1:10	IL-2 10 IU/ml IL-12 1ng/ml anti-IL-4 10 μ g/ml	IL-2 10 IU/ml IL-12 1ng/ml
Th2	Naïve CD4 ⁺ T-cells	1:10	IL-2 10 IU/ml IL-4 20ng/ml anti-IL-12 10 μ g/ml anti-IFN- γ 10 μ g/ml	IL-2 10 IU/ml IL-4 20ng/ml
Th17	Memory CD4 ⁺ T-cells	1:50	IL-1- β 10ng/ml IL-23 10ng/ml TGF- β 10ng/ml	IL-2 10U/ml IL-23 10ng/ml
Tfh	Total CD4 ⁺ T-cells	1:10	IL-12 10 ng/ml IL-6 10 ng/ml IL-1 β 10 ng/ml TGF- β 10 ng/ml	IL-12 10 ng/ml IL-6 10 ng/ml IL-1 β 10 ng/ml TGF β 10 ng/ml

Table 3. Conditions for the differentiation of T-helper cell subsets. Table showing the conditions for T-helper differentiation including cell type used, bead: cell ratio and cytokines used.

400g for 7 minutes. Cells were counted and plated out at a concentration of 1×10^6 cells/ml. Tfh cells were rested for 24 hours in the absence of IL-2, which has been shown to inhibit Tfh differentiation (442, 443). Th1, Th2 and Th17 cells were rested for 4 days with addition of 10 U/ml IL-2 (Roche) per well. Cells incubated at 37°C, 5% CO₂. After 1 or 4 days rest cells were further stimulated with PMA/ionomycin to assess intracellular cytokines (as described in *Chapter 2.9.3*).

2.8 Measurement of Proliferation

Proliferation of total CD4⁺ T-cells, naïve and memory CD4⁺ T-cells was measured in one of two ways: by CFSE labelling followed by flow cytometry or by tritiated thymidine incorporation assay.

2.8.1 CFSE labelling

Proliferation of cells can be tracked by use of 5, 6-carboxyfluorescein diacetate succinimidyl ester (CFSE). This dye is able to cross intact cell membranes where its acetate groups are cleaved by intracellular esterase to give fluorescent carboxyfluorescein. The dye is able to form crosslinks with intracellular proteins by interaction between succinimidyl ester and primary amines. Cell divisions can be tracked by the halving in fluorescence intensity, which can be monitored using flow cytometry.

Freshly isolated total CD4⁺ T-cells, naïve and memory CD4⁺ T-cells were centrifuged at 400g for 7 minutes and resuspended in 14ml PBS. Cells were centrifuged at 600g for 7 minutes, washed with PBS and then centrifuged at 600g for 10 minutes. Cells were then incubated for 5 minutes at 37°C with 1µM CFSE (eBioscience) at a ratio of 1:1, giving a final concentration of 0.5 µM. Staining was terminated by adding 15ml RF10 + 10% FCS, which will bind to any remaining CFSE, and incubating on ice for 5 minutes. Cells were then centrifuged twice at 600g for 7 minutes and resuspended in culture media before being counted and cultured.

2.8.2 Tritiated thymidine incorporation assay

Tritiated thymidine (³H-TdR) is a radioactive nucleotide that is added to the cell culture. This molecule will then be incorporated into newly synthesised strands of DNA by binding to adenosine, allowing the cell divisions to be quantified.

Following stimulation in an MLR or with anti-CD3 and anti-CD28 as described in *Chapter 2.7.3-4*, cells were incubated with 20µl ³H-TdR (Perkin Elmer; Massachusetts, USA) for 8 hours at 37°C with 5% CO₂. Cells were then harvested onto filters and counted with a 1450 micro-B trilux, microplate scintillation and luminescence counter (Perkin Elmer). Results are given as counts per minute (CPM).

2.9 Flow Cytometry

Flow cytometry is the measurement of cell characteristics using light. Cells are passed singly through a laser, which causes light to scatter, and fluorescence of differing wavelengths can be produced, which is detected by the optics system and recorded. Light is turned into numerical values by the electronics system and results are displayed visually as dot plots. The amount and the direction of light scatter indicate the cells size (forward scatter; FSC-A) and granularity (side scatter; SSC-A). Multiple parameters can also be measured including cell surface markers, intracellular cytokines, proliferation and viability by the use of fluorescently labelled antibodies or dyes which have specific wavelengths of absorption.

2.9.1 Cell surface markers

To measure parameters such as cell surface markers mAbs to specific antigens can be conjugated to fluorescent dyes (fluorochromes) which emit fluorescent light when excited by lasers. These antibodies can be used to give knowledge of cell phenotype.

Freshly isolated or cultured cells, 0.2×10^6 - 1×10^6 , were resuspended in 200 μ l fluorescence active cell sorting (FACS) buffer (*Chapter 2.1*), transferred to 96 well v-bottom plate, and further centrifuged at 400g for 3 minutes. Cells were subsequently incubated for 30 minutes in the dark at 4°C with FACS buffer, hIgG (gift from Dr. Sophie Hambleton, Newcastle University) at 4 μ g/ml to block non-specific binding of antibodies to Fc receptors. Antibodies were added with the dilution determined by titration of the antibody on 1×10^6 PBMC by members of the Musculoskeletal Research Group (*Table 4*). Cells were then washed twice with FACS buffer and centrifuged at 400g for 3 minutes, prior to acquisition.

Cells were either acquired immediately or resuspended in FACS buffer with 1% formaldehyde and acquired within one week of staining. Formaldehyde is able to fix cells by forming crosslinks between proteins in the cell.

Surface stain	Antibody	Fluorophore	Clone	Species	Isotype	Dilution	Company
CD4 Purity	CD3	PB	UCHT1	Mouse	IgG1	1:50	BD Biosciences
	CD4v4	FITC	L120	Mouse	IgG1	1:10	BD Biosciences
	CD14	PE	M5E2	Mouse	IgG2a, κ	1:20	BD Biosciences
	CD19	APC	HIB19	Mouse	IgG1, κ	1:10	BD Biosciences
Naïve Purity	CD3	PB	UCHT1	Mouse	IgG1	1:50	BD Biosciences
	CD4	FITC	RPA-T4	Mouse	IgG1, κ	1:50	Biolegend
	CD14	PE	M5E2	Mouse	IgG2a, κ	1:20	BD Biosciences
	CD19	APC	HIB19	Mouse	IgG1, κ	1:10	BD Biosciences
Phenotype	CD45RA	PerCpCy5.5	HI100	Mouse	IgG2b, κ	1:50	Biolegend
	CD3	BV510	UCHT1	Mouse	IgG1, κ	1:50	Biolegend
	CD4	APCeFluor780	SK3	Mouse	IgG1, κ	1:50	eBioscience
	CD25	PECy7	BC96	Mouse	IgG1, κ	1:50	Biolegend
	CD27	V450	M-T271	Mouse	IgG1, κ	1:50	BD Biosciences
	CD28	APC	CD28.2	Mouse	IgG1, κ	1:10	BD Biosciences
	CD45RA	PerCpCy5.5	HI 100	Mouse	IgG2b, κ	1:50	Biolegend
	CD126 (IL-6R)	CFS	17506	Mouse	IgG1	1:10	R & D Systems
	CD130 (gp130)	PE	2E1B02	Mouse	IgG2a, κ	1:10	Biolegend
	CD130 (gp130)	PE	2E1B02	Mouse	IgG2a, κ	1:10	Biolegend
Activation	CD3	BV510	UCHT1	Mouse	IgG1, κ	1:50	Biolegend
	CD4	APCeFluor780	SK3	Mouse	IgG1, κ	1:50	eBioscience
	CD25	PECy7	BC96	Mouse	IgG1, κ	1:50	Biolegend
	CD154	PE	24-31	Mouse	IgG1, κ	1:10	Biolegend
	CD185	AF647	J252D4	Mouse	IgG1, κ	1:10	Biolegend
	CD185	AF647	J252D4	Mouse	IgG1, κ	1:10	Biolegend

Table 4. Flow cytometry antibodies used for purity, phenotype and activation analysis. Table shows fluorophore, clone, dilution and source of each antibody. CD4v4 is a different clone recognising amino acids 306-370 of the CD4 antigen which are not masked by the binding of CD4 antibody complexes in the positive selection of CD4⁺ T-cells.

2.9.2 Viability staining

Annexin V and 4', 6-diamidino-2-phenylidone (DAPI) staining is a common method of assessing viability and gives information regarding the stages of apoptosis. DAPI is a dye that can cross the membrane of only dead cells. Annexin V is a protein, conjugated to a fluorochrome, which is able to bind phospholipid phosphatidylserine (PS) residues typically found on the inner cytoplasmic membrane. These residues are translocated to the outer cytoplasmic membrane in the early process of apoptosis to signal that the cell can be phagocytosed. The combination of these two viability markers aids analysis by allowing the identification of viable, early/late stages of apoptosis and dead cells. Zombie aqua is a similar viability dye that crosses the cell membrane, showing viable and dead cells.

As a measure of viability freshly isolated and cultured naïve CD4⁺ were harvested and washed with FACS buffer as previously described. Cells were resuspended in binding buffer (*Chapter 2.1*) prior to addition of Annexin V APC (Biolegend; 1:10) and 3µM DAPI (Life Technologies; California, USA). Cells were incubated in the dark for 15 minutes at room temperature prior to acquisition.

Alternatively, cultured cells were harvested and washed in PBS (Lonza) as previously described. Cells were resuspended in 50µl PBS and 0.5µl zombie aqua (Biolegend) and incubated in the dark for 15 minutes at room temperature. Cells were washed and resuspended in FACS buffer for acquisition or subsequent cell surface staining.

2.9.3 Intracellular cytokines

In order to detect intracellular cytokines naïve or memory CD4⁺ T-cells were stimulated in a non-specific manner with PMA and ionomycin. PMA diffuses across cell membranes to activate protein kinase C and ionomycin triggers intracellular calcium release, which together activates cells and triggers cytokine production as resting cells only produce a minimal level of cytokine. Brefeldin A (BFA) is then used to retain the cytokines in the cell by preventing formation of transport vesicles and stopping protein transport from the endoplasmic reticulum (ER) to the Golgi apparatus. Cells must then be permeabilised to allow staining with anti-cytokine antibodies.

To cells cultured for intracellular cytokine staining 10ng/ml PMA (Sigma Aldrich) and 1µg/ml ionomycin (Sigma Aldrich) were added to cells and incubated at 37°C for 1 hour. After 1 hour BFA (Sigma Aldrich) was added at 1mg/ml and cultured for a further 4 hours at 37°C (unless otherwise stated). Control wells with BFA only were also added in order to carry out gating.

Cells for staining were harvested, resuspended in 200µl FACS buffer transferred to 96 well v-bottom plate and centrifuged at 400g for 3 minutes. Cells were subsequently incubated for 30 minutes in the dark at 4°C with FACS buffer, hIgG and antibodies for surface stain (*Table 6*). Cells were washed twice with FACS buffer as previously described. Cells were then incubated in Foxp3 fixation/permeabilisation buffer (eBioscience) for 30 minutes in the dark at 4°C. This buffer fixes the cells via formaldehyde but also permeabilises cells using saponin, allowing antibodies to bind intracellular proteins. Cells were then washed twice with FACS buffer before resuspending in FACS buffer to be stored overnight at 4°C in the dark.

Within a week of surface staining cells were centrifuged at 400g for 3 minutes and incubated in 150µl Foxp3 permeabilisation buffer (eBioscience) for 15 minutes in the dark at 4°C. Cells were centrifuged 400g for 3 minutes and incubated for in the dark at 4°C for 15 minutes with 50µl permeabilisation buffer and 2% mouse serum (Sigma Aldrich) to block non-specific binding. Subsequently, antibodies for intracellular cytokine staining or transcription factor staining were added (*Table 5*) and incubated for 30 minutes, as before. Cells were then washed twice with permeabilisation buffer and resuspended in FACS buffer for subsequent acquisition.

Intracellular staining	Antibody	Fluorophore	Clone	Species	Isotype	Dilution	Company
Cytokines	CD3 (surface)	PB	UCHT1	Mouse	IgG1	1:50	BD Biosciences
	CD4 (surface)	PerCP	SK3	Mouse	IgG1, κ	1:20	BD Biosciences
	IFN-g	FITC	4S.B3	Mouse	IgG1, κ	1:100	EBiosciences
	IL-4	PeCy7	8D4-8	Mouse	IgG1, κ	1:200	EBiosciences
	IL-17	APCeF660/ APCCy7	64DEC17/BL 168	Mouse	IgG1, κ	1:10	EBiosciences/ Biolegend
	IL-21	PE	EBIO3A3-N2	Mouse	IgG1, κ	1:10	EBiosciences
Transcription factors	CD3	PerCP	SK7	Mouse	IgG1, κ	1:20	BD Biosciences
	CD4	APCeF780	SK3	Mouse	IgG1, κ	1:50	EBiosciences
	T-bet	BV421	O4-46	Mouse	IgG1, κ	1:20	BD Biosciences
	GATA-3	eF660	TWAJ	Mouse	IgG2b, κ	1:20	EBiosciences
	ROR-γt	AF488	Q21-559	Mouse	IgG2b, κ	1:20	BD Biosciences
	Foxp3	PE	236A/E7	Mouse	IgG1, κ	1:20	BD Biosciences

Table 5. Flow cytometry antibodies used for intracellular cytokine staining. Table includes fluorophore, clone, dilution and source of each antibody.

2.9.4 Whole blood phenotype

Whole blood staining for flow cytometry is the process of staining blood with fluorophore-conjugated mAbs to specific antigens on cells in the blood. This process involves the addition of lysing solution to remove red blood cells and fix white blood cells. As cell processing can introduce variability whole blood was used to analyse the expression of IL-6 signalling receptors gp130 and IL-6R on CD4⁺ T-cell subsets (true naïve, central memory and effector memory, *Figure 9*) from early arthritis patients.

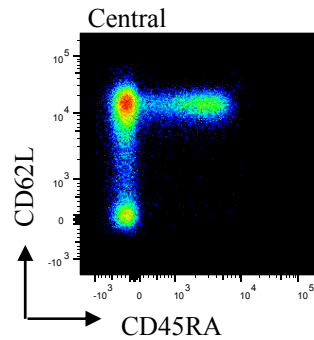


Figure 9. Identification of true naïve, central memory and effector memory CD4⁺ T-cells. CD4⁺ T-cells are gated as CD3⁺CD4⁺ and from this T-cell subsets are identified by their differential expression of CD45RA and CD62L. True naïve CD4⁺ T-cells are CD45RA⁺CD62L⁺, central memory CD4⁺ T-cells are CD45RA⁻CD62L⁺ and effector memory CD4⁺ T-cells are CD45RA⁺CD62L⁻.

200µl EDTA fresh whole blood of patients from the Early Arthritis Clinic was incubated with antibodies for whole blood staining (*Table 6*) for 30 minutes at 37°C. Samples were then incubated with 10x volume of FACS lysing solution (BD Biosciences; Oxford, UK) for 12 minutes at 37°C. Cells were then washed once in FACS buffer, prior to acquisition.

Whole blood stain	Antibody	Fluorophore	Clone	Species	Isotype	Dilution	Company
Phenotype	CD3	BV510	UCHT1	Mouse	IgG1, κ	1:20	Biolegend
	CD4	APCeFluor780	SK3	Mouse	IgG1, κ	1:200	eBioscience
	CD27	V450	M-T271	Mouse	IgG1, κ	1:200	BD Biosciences
	CD28	APC	CD28.2	Mouse	IgG1, κ	1:50	BD Biosciences
	CD45RA	FITC	HI 100	Mouse	IgG2b, κ	1:200	BD Biosciences
	CD62L	PECy7	DREG-56	Mouse	IgG1, κ	1:100	Biolegend
	CD126	PerCPeFluor710/ CFS	47.797.1F2/ 17506	Mouse	IgG1	1:50	eBioscience
	CD130	PE	2E1B02	Mouse	IgG2a, κ	1:50	Biolegend

Table 6. Flow cytometry antibodies used in whole blood phenotype staining. Table includes fluorophore, clone, dilution and source of each antibody.

2.9.5 Data acquisition and analysis

Cells were analysed using FACS Canto II (BD Biosciences) and compensated using compensation beads (BD Biosciences) bound to the antibody of interest.

Subsequent data was analysed using FlowJo version 10 (Treestar Inc.; Oregon, USA). To ensure the correct placement of gates internal negative controls were used in whole blood assays, with gates set based on other populations in the sample which do not express the marker of interest. In experiments where the starting population of CD4⁺ T-cells were available, as in purity and phenotype analyses, gates were set on this population and transferred to the population of interest, namely naïve or memory CD4⁺ T-cells. The disadvantage to this method is that with purified population the antibody to antigen ratio can decrease if not enough antibody is added compared to the starting population resulting in a left shift. Further optimisation of the antibody would be required for use on purified populations. For intracellular staining gates were set on BFA only controls (i.e. unstimulated) and then applied to the population of interest.

In some cases, there was clear bimodal expression of a particular antigen resulting in clear positives and negatives meaning gates could be easily determined. In other cases, gates were set based in an unstained population of cells and in the case of CD40L; gates were set on fluorescence minus one (FMO) controls. The use of unstained cells takes into account the background fluorescence and autofluorescence of the sample and gates can be set to determine the negative populations. An FMO control includes all of the antibody conjugates present in the test sample except one. The advantage of an FMO control is that it can help identification of a positive population when the expression of that marker is low. This method allows the assessment of the spread of fluorescence as a fluorophore from another may leak into the channel of interest causing false positive staining. This approach however does not provide information on background non-specific staining which can be tested for by the use of isotype controls. Isotype controls ensure that observed staining is due to the specific binding of antibody to its target and not due to non-specific binding to Fc receptors or cellular components. However, the ratio of fluorophore to antibody must be the same as the antibody of interest, which can vary greatly between reagents, creating variable levels of background staining. These controls also do not account for fluorescence spill over from other channels (444). For this reason isotype controls were not used in this thesis.

Proliferation was calculated using the proliferation tool on FlowJo. This tool calculates division index (DI), proliferation index (PI) and the percent divided (% divided) for each sample. These provide information on cell divisions and numbers of cells entering division:

- DI – The average number of cell divisions a cell in the original population has undergone (includes undivided cells).
- PI – Total number of divisions divided by the number of cells that went into division (includes only divided cells).
- Percentage divided – Precursor frequency of dividing cells (the probability of a cell dividing).

An example of the gating strategy for proliferation analysis can be seen in *Figure 10*, all other gating strategies can be found in *Figures 1-8* in the *Appendix*.

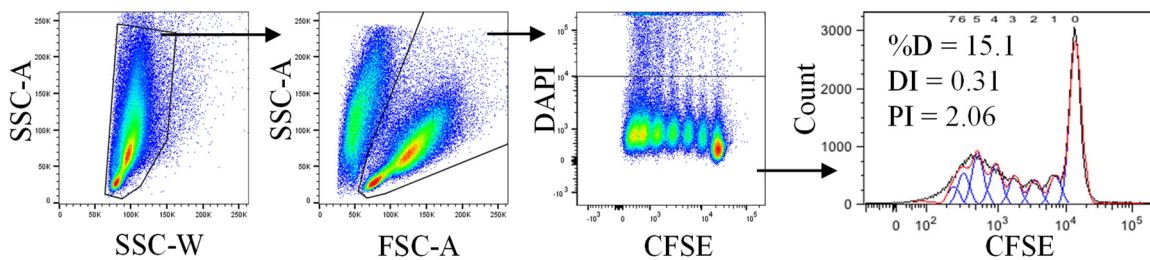


Figure 10. Gating strategy for proliferation analysis. CFSE labelled naïve and memory CD4⁺ T-cells were stained with DAPI before acquisition on the flow cytometer. Cells were gated on SSC-A v SSC-W to exclude debris followed by SSC-A v FSC-A to gate lymphocytes. Proliferating cells were then gated as DAPI negative and proliferation analysed using FlowJo proliferation tool.

2.10 Cytokine Detection

A sandwich enzyme linked immunosorbent assay (ELISA) or MesoScale Discovery (MSD) allows quantitative measurement of substances such as peptides within a known range.

In the case of an ELISA, the capture antibody, toward the antigen of interest, must be immobilised onto 96-well high affinity EIA/RIA flat bottom plates. Non-specific binding sites are blocked so the antibody captures the antigen within the sample. A biotinylated detection antibody binds to the antigen of interest, followed by a streptavidin conjugated enzyme label which binds the biotin of the detection antibody. A substrate is added which once bound to the enzyme can be converted to absorbance signal to indicate antigen quantity. This can be compared to a standard curve of known concentrations.

The MSD is a similar technique using the “sandwich principle”, however, plates are bought pre-coated with up to 16 antibodies of interest and detection occurs using electrochemoluminescence. This works as high binding carbon electrodes in the microplates attach biologic reagents. Electrochemiluminescent labels are conjugated to detection antibodies, when voltage is applied to the plate electrodes light is emitted from these labels, and light intensity can be quantified.

Supernatants from cell cultures were frozen at -20°C at the time of harvest for detection of IL-10, IFN- γ , IL-4 and IL-5. Serum from healthy donors and EAC attendees were taken at baseline. Serum was separated by centrifuging 1200g for 12 minutes at room temperature and then frozen at -20°C. Samples were defrosted for use in ELISA or MSD.

2.10.1 Sandwich Enzyme Linked Immunosorbent Assay

96 well EIA/RIA flat bottom plates (Costar, Corning) were coated with IL-10, IFN- γ , IL-4, IL-5 (BD biosciences) or IL-17 (eBiosciences) capture antibody diluted in phosphate buffer (*Chapter 2.1*) as per manufacturers instructions, overnight at 4°C, *Table 7*. Plates were then washed 3 times with ELISA wash buffer (*Chapter 2.1*) then blocked with 100 μ l ELISA blocking buffer for 2 hours at room temperature. Plates were then washed 5 times in wash buffer. Cell supernatants (diluted to 1:2 and 1:10 with diluent (*Chapter 2.1*)) and standards (top standard with 2-fold serial dilution) were added to the plate and incubated overnight at 4°C. After washing plates as before biotinylated detection antibody was added diluted in diluent (final concentrations in *Table 7*) and incubated at room temperature for 1 hour. Plates were washed again and 50 μ l of streptavidin-horseradish peroxidase (HRP; Extr Avidin; Sigma Aldrich) diluted 1/1000 in diluent was added and incubated at room temperature in the dark for 30 minutes. Plates were washed before addition of ortho-phenylenediamine dihydrochloride substrate (OPD; Sigma Aldrich; Missouri, USA), diluted in 13ml citrate phosphate buffer with H₂O₂ (6 μ l 30% stock, added immediately prior to use) and plates incubated for 30 minutes in the dark, or until colour developed. Reaction was stopped by adding 3M H₂SO₄ and optical density was read at 450nm or 490nm (*Table 7*) on a Sunrise micro-plate reader (Tecan Group Ltd; Mannedorf, Switzerland). A standard curve of absorbance can be plotted using known concentrations for the cytokine of interest as in *Figure 11*. Samples were analysed in duplicate and a line of best fit is then applied using Microsoft Excel. The concentration of the cytokine of interest can then be calculated for samples using the equation $y=mx+c$ and R^2 .

Cytokine	Capture Antibody (µg/ml)	Detection Antibody (µg/ml)	Top Standard (pg/ml)	Developing Time (mins)	Absorbance (nM)
IFN-γ	1	1	2000	20-30	490
IL-5	0.5	0.5	3000	30	490
IL-10	2	1	2000	30-40	490
IL-17	1/250	1/250	500	15	450

Table 7. The concentration of antibodies used for ELISA. Table includes concentrations of capture and detection antibodies, concentration of the top standard, developing time of the assay and the absorbance of each assay.

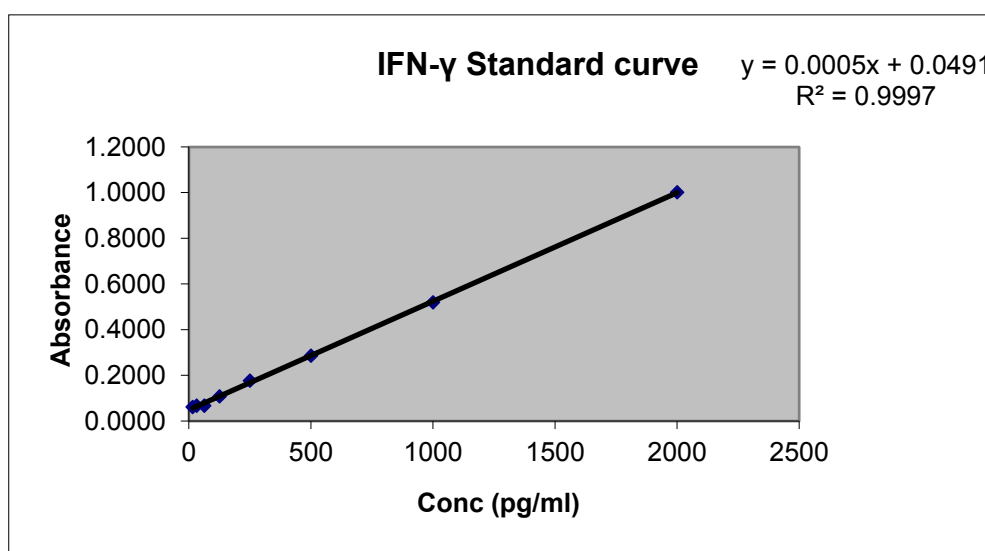


Figure 11. Example of IFN-γ standard curve in ELISA analysis. Standard curve plotted using known concentrations of the cytokine of interest and line of best fit is applied.

2.10.2 MSD

MSD analysis of supernatants was carried out according to manufacturer's instructions. Briefly, calibrators of known concentration for IL-17 and tenplex cytokines were made with 4-fold dilutions from 10000pg/ml to 0pg/ml. 25µl of samples were added to IL-17 plate or 50µl 1:1 samples diluted with diluent (Meso Scale Discovery; Maryland, USA) were added to tenplex plates. Plates were sealed and incubated for 2 hours at 500rpm. Plates were washed 3 times in PBS + 0.05% tween 20 and 25µl diluted detection antibodies were added. Plates were sealed and incubated for 2 hours with shaking. Plates were washed 3 times as before and 150µl read buffer, diluted 1:1, was added. The plate was then analysed plate on SECTOR imager (Meso Scale Discovery). The dynamic range for each cytokine measured can be seen in *Table 8*.

Cytokine	Dynamic Range (pg/ml)
IFN- γ	0.20-938
IL-1 β	0.04-375
IL-2	0.09-938
IL-4	0.02-158
IL-6	0.06-488
IL-8	0.04-375
IL-10	0.03-233
IL-12p70	0.11-315
IL-13	0.24-353
TNF- α	0.04-248
IL-17	0.4-10000

Table 8. Dynamic range of cytokine detection of the MSD plate. Table showing cytokines used in MSD analysis and the dynamic range of detection for each cytokine.

2.11 Quantification of Gene Expression

For the quantification of gene expression RNA is extracted from cells under different experimental conditions. Samples are first lysed to disrupt cell walls and homogenised to shear the DNA and other cell components. Samples are then applied to spin columns which binds RNA and contaminants are washed away. Additional DNase treatment removes any residual DNA. Total RNA can then be eluted, quantified and reverse transcribed into complementary DNA (cDNA). First RNA is denatured with the help of reducing agent dithiotheritol (DTT) which breaks disulphide bonds to generate single stranded RNA. The cDNA is then made, a process involving annealing and extension, random hexamers act as primers and

deoxynucleoside triphosphates (dNTPs) provide the nucleotides for the synthesis of cDNA which is driven by superscript II reverse transcriptase enzyme.

Following reverse transcription cDNA is amplified and quantified by TaqMan real time PCR. This technique works as an initial high temperature allows the denaturation of double stranded cDNA. The temperature is then lowered which allows the binding of primers, short sequences against the gene of interest. Temperature is then raised again to drive a thermostable polymerase enzyme which is able to synthesise the complementary sequence. This cycle creates twice the copy number and is carried out for a total of 40 cycles. In the TaqMan real time PCR reaction this process is monitored by the detection of fluorescence which is provided by probes. Probes are DNA oligonucleotides which have a fluorescent reporter molecule at the 5' end and a quencher molecule at the 3' end. The interaction of these molecules mean fluorescence is suppressed. Probes bind downstream of primers and are then cleaved by the polymerase enzyme to separate the reporter and the quencher allowing fluorescence at a certain wavelength. This level of fluorescence correlates with the level of DNA amplification to allow quantification.

Housekeeping genes are genes which are constitutively expressed by a certain cell which do not change under differing experimental conditions such as RNA polymerase II subunit A (POLR2A, RPB1), a polymerase responsible for synthesising mRNA, and 18S, a ribosomal RNA. These genes are used as a reference point for analysis of expression levels of the gene of interest in order to normalise data. This allows a ratio for the expression of genes of interest divided by the expression of the normalised housekeeper gene to allow comparison between samples without the knowledge of absolute expression.

2.11.1 RNA homogenisation

CD4⁺ T-cells at 1×10^6 were harvested, washed with Hanks + 1% FCS and centrifuged at 400g for 7 minutes. Cells were then homogenised in RLT buffer (Qiagen; Hilden, Germany) supplemented with 10% β -metacaptotoethanol (Sigma Aldrich). Cells were vortexed and transferred to QIA shredder columns (Qiagen) before being centrifuged at 13000rpm for 2 minutes. Shredder columns were removed and homogenates stored at -80°C for maximum of 3 months.

2.11.2 RNA Extraction

RNA was then isolated using Qiagen RNeasy mini kit according to manufacturer's protocol. To the homogenised lysate 1 volume of 70% ethanol (Fisher Scientific) was added, transferred to an RNeasy spin column, centrifuged for 15 seconds at 8,000 g and flow-through was discarded. Cells were then washed once with 500 μ l of buffer RPE and centrifuged for 15

seconds at 8,000 g. Cells were washed again with 500µl of buffer RW1 and centrifuged for 15 seconds at 8,000 g before being incubated with DNase I for 15 minutes. Cells were then washed as previously described with RW1 followed by RPE. Cells were then placed in a new RNeasy column and 30µl RNase-free water (Qiagen) was added and centrifuged for 1 minute at 10,000 rpm.

Using a Nanodrop 1000 UV Spectrophotometer (Thermo Fisher Scientific) RNA was quantified and quality assessed according to manufacturer's instructions. Concentrations and 260:280 ratios, which provide a measure of nucleic acid quality, are given in the *Appendix*. Typically, a concentration of 1×10^6 cells gave a median yield of 29.75ng/µl RNA with a median 260:280 ratio of 1.905.

2.11.3 Reverse Transcription

cDNA was synthesised from RNA (in duplicate) using Superscript II Reverse Transcriptase (Invitrogen). A reverse transcriptase free sample and water sample were also used as negative controls.

To all samples 1µg/µl random hexamers (Thermo Fisher Scientific) was added and incubated for 10 minutes at 70°C on thermal cycler, followed by a 10 minute incubation on ice. To each of the samples 4µl 5x RT buffer (Thermo Fisher Scientific), 2µl 0.1M DTT (Invitrogen), 0.5µl 10mM dNTP (Invitrogen), 0.5µl Superscript II and 2µl H₂O was added and incubated for 1 hour at 42°C, *Table 9*.

Stage of Reverse Transcription	Thermocycler Setting
Denaturation	70°C, 10 minutes
cDNA synthesis	42°C, 1 hour

Table 9. Thermocycler settings for reverse transcription. Table showing the stage of reverse transcription with the temperature and length of incubation on the thermocycler.

2.11.4 TaqMan real time Polymerase Chain Reaction

After reverse transcription, cDNA was transferred to 96 well PCR plates in duplicate (Starlab; Brussels, Belgium) and then amplified using TaqMan assay (Applied Biosystems; California, USA). For genes of interest and POLR2A, RPB1 housekeeping gene, cDNA was diluted 1 in 2.5, with water (Sigma Aldrich). To cDNA universal FAM probes, forward and reverse primers (*Table 10*) were added at 10µM along with 10µl of TaqMan Gene Expression Master Mix (2x; Applied Biosystems). For the housekeeping gene, 18S, cDNA was diluted 1 in 100, in-house FAM-TAMRA probe was used at 15µM, forward and reverse primers were used at 30µM and

4.7µl of TaqMan Gene Expression Master Mix (2x) were added. PCR settings can be seen in *Table 11* and included 1 cycle of denaturation at 90°C for 1 minute, 45 amplification cycles at 95°C for 15 seconds, 55°C for 15 seconds and 72°C for 10 seconds. Raw gene expression data was normalized to the 18S or POLR2A housekeeping genes using the calculation $2^{-\Delta CT}$ values, where ΔCT represents $CT(\text{target gene}) - CT(\text{housekeeping gene})$.

Gene	Forward Primer	Reverse Primer	Probe
18S (NR_003286)	cgaatggctcattaaatcagttatgg	tattagctctagaattaccacagttatcc	tcctttggtcg ctcgcctcctc
POLR2ARPB1 (NM_000937.4)	ttgtgcaggacacactcaca	caggagggtcatcacttcacc	UPL 1
BCL-3 (NM_005178)	cgacatctacaacaacctacgg	ccacagacggtaatgtgggtg	UPL 39
SOCS-3 (NM_003955)	agacttcgattcgggacca	aacttgctgtgggtgacca	UPL 36
PIM-1 (NM_002648)	gatttccgactggggagag	agtccaggagcctaatacg	UPL 18
BCL-6 (NM_001706)	ttccgctacaagggaac	tgcaacgatagggtttctca	UPL 5
REXO2 (NM_015523)	aaggagcatcacgggaagt	tcgtacaaaggacagaaattcatact	UPL 40
SBNO2 (NM_014963)	gactacgtgccgtccaagtc	cagggtgtaggtgatgtctgg	UPL 54
LRP8 (NM_004631)	actcccgaagaacctcttt	caaggcttaatgccactcg	UPL 27
C10orf10 (NM_007021)	gtccctgctcatccattctc	cactctggcgagaggaggt	UPL 1
CST7 (NM_003650)	ctctcaggcctctgacgagt	ttaggaggtgctaccatgctg	UPL 59
GPT2 (NM_001142466)	gatcctgcaggcttgtgg	ttcacggatgcagttgacac	UPL 64
RRAD (NM_001128850)	gacgagagcggttacaagggtg	ggagcgatcataggtgtgc	UPL 3
CBS (NM_001178008)	atcatttcccctcccgtct	cagagacacaaagtcgatcagc	UPL 52
GSTO1 (NM_004832)	gcacttttgagctaaggaggaa	aggggattcaggaagtaggtg	UPL 12

Table 10. Nucleotide sequences of forward and reverse primers and probes for each gene used in TaqMan real time PCR assays. Probes from Universal Probe Library (Roche).

Stage of RT-PCR	Thermocycler Setting	Number of Cycles
Glycosylase activation	50°C, 2 minutes	1
Polymerase activation	95°C, 10 minutes	40
Denaturation	95°C, 15 seconds	40
Annealing and Extension	60°C, 1 minute	40

Table 11. Thermocycler settings for TaqMan real time PCR. Table showing the stage of real time PCR with the temperature and length of each cycle.

2.11.5 Real time PCR analysis

RT-PCR was carried out using the Aria Mx Real-Time PCR system (Agilent Technologies, California, US). A graph was produced of fluorescence intensity versus cycle number on a log scale. Thresholds were set for each gene at the point where the fluorescence curve begins its exponential phase. CT values were then analysed in Excel (Microsoft Office). Delta CT can be calculated by subtracting gene of interest CT value from the selected housekeeping gene CT value. The housekeeping gene chosen for cytokine stimulation experiments, *Chapter 4.3.1*, was 18S as this is a commonly used housekeeping gene, which had been previously established in the lab to be consistent across CD4⁺ T-cells in different experimental conditions (Dr Pratt, personal communication). For the analysis of gene expression of early arthritis patients compared to healthy controls, *Chapter 6.3.3-4*, housekeeping gene POL2RPB1 was tested against the 18S housekeeping gene. For these experiments, the CT values of both the 18S and the POL2RPB1 housekeeping genes were measured and the CT values of the POL2RPB1 gene were found to be most comparable to the CT values of the genes of interest and were more stably expressed so this was chosen in order to allow for fairer comparisons (data not shown). The relative quantity of mRNA is $2^{-\Delta CT}$. Fold induction could be analysed by comparing cytokine-stimulated conditions with untreated conditions at each time point.

2.12 Microarray

Microarray is a high throughput method of measuring gene expression levels of a large number of genes in RNA samples simultaneously. The Illumina microarray uses bead-based technology to provide gene expression profiling. This method involves 3-micron silica beads, which are attached to oligonucleotide probes and immobilised by random distribution in microwells. These oligonucleotide probes are 79 nucleotides in length and each bead has a 29 base identifiable “address” probe to identify the location of each bead. Multiple copies of each bead may be present in one well. This can be used as technical replicate for variance stabilising transformation. The remaining 50 nucleotides are specific for gene sequences and detect

labelled cRNA. This provides high sensitivity and highly reproducible gene expression quantification with reduced sample-to-sample variability.

RNA was extracted as described in *Chapter 2.11* from naïve and memory CD4⁺ T-cells of 4 biological replicates. RNA was then shipped on dry ice to Tepnel Pharma Services, where samples were processed into double stranded cDNA, amplified to create cRNA and hybridised to Illumina Human HT-12 v4.0 microarray. The Human HT-12 v4.0 Expression BeadChip allows human genome wide expression profiling of well-characterised genes, gene candidates and splice variants. Each array contains probes for over 47,000 transcripts.

Briefly, total RNA was reverse transcribed to synthesise single-strand cDNA. This was converted to double stranded DNA to provide the template for transcription. cDNA is then purified before being amplified and labelled generating a pool of biotin labelled cRNA. This cRNA is then purified and quantified for use on the assay. Equivalent amounts of labelled RNA is loaded into BeadChips, which are then placed in hybridisation chambers and incubated for 14-20 hours at 58°C. cRNA is hybridised to each array on the BeadChip, which contains the complementary gene sequence. The BeadChip is then washed numerous times before being blocked. Finally, Cy3-Streptavidin is introduced to the array, which binds the analytical probes hybridised to the BeadChip, allowing differential detection of signals upon scanning. The Illumina HiScan or BeadArray Reader, a high-resolution imager, scans the BeadChip at a particular wavelength and light emissions are recorded as high-resolution images. The fluorescence intensity at each bead location can be determined and the intensity of the signal is proportional to the quantity of transcript in the original sample. Data files of this information are created and GenomeStudio software is then used to export probe summary profiles as files, which allows further analysis of the data. The raw data of intensities was securely transferred electronically in the form of IDAT files, which were used for analysis.

Controls included housekeepers, genes known to be constitutively expressed to ensure the biological specimen remained intact, RNA Spike, the introduction of known concentration of RNA to ensure correct sample labelling, Cy3-labelled hybridisation controls, which test the hybridisation of assay products to the array beads, Biotin control, to monitor signal generation and negative controls to determine the background of the system and any signal resulting from non-specific binding or cross hybridisation.

2.12.1 Microarray analysis

Prior to receiving data background correction was carried out in GenomeStudio (Illumina Inc., San Diego). Probe profiles were read into R software version 3.3.0 and all data analysis was

carried out in the R software environment, a free open-source software for statistical computing. Data was analysed using the Bioconductor and limma packages, add-on modules that provide the tools for analysis of high-throughput genomic data. Code for data analysis on R was carried out in dialogue with a bioinformatician via collaboration with Newcastle University's core Bioinformatics Support Unit and the author carried out further analysis.

Microarray data was processed, including quality control, normalisation and gene annotation, using the lumi package in Bioconductor (445, 446). Data quality was assessed by visualising raw data as box-plots to assess background intensities, to highlight whether any arrays had unusually high background intensities. Within-assay normalisation was performed using variance stabilising transformation (VST) (447). This uses within-array technical replicates to reduce false positives. Following this between array normalisation was carried out using robust spline normalisation (RSN), which ensures intensity values across arrays have the same distribution using a combination of quantile normalisation and Loess normalisation (446). At this stage probes can be removed which fail to pass detection threshold and transformation and normalisation procedures repeated. Data was then subject to principle component analysis (PCA) and plots were generated which plot the 2 measures which account for the greatest variance in the data and arrays whose gene expression is similar will cluster together (448). As probes are specific to the technology used these need to be matched to external identifiers such as nuID's to allow for cross platform data integration (449).

The limma package is used for further differential gene expression analysis, which uses Empirical Bayesian methods to moderate variance of log fold changes across arrays (450). A design matrix was created and a linear model fitted to each gene. Comparisons can then be made by fitting to the linear model and applying empirical Bayes statistics to obtain differential expression values for all probes. The final stage of analysis involves filtering of differential expression lists by applying log fold change and p-value cut offs and applying multiple test correction to test statistics, which controls for error rate giving a lower number of false positives. Data was analysed with an adjusted p-value cut off of $p < 0.05$ and a log fold change of 1.5 and multiple test correction carried out using the Benjamini-Hochberg method (451). Differential gene induction was visualised using volcano plots and the ggplot2 package. Lists of differentially expressed genes, Gene Lists 1A-5D, can be found in CD-ROM in the *Appendix*.

Further tests used in the analysis of gene expression data include hypergeometric testing which is a statistical test to determine the probability of genes differentially expressed between early RA and disease controls occurring in IL-6 exposed naïve CD4⁺ T-cells. Functional pathway analysis was carried out using Ingenuity Pathway Analysis (Qiagen). This is a web-based

software for analysis of microarrays. It can provide information on genes and proteins and build models to explore experimental systems. Information is provided in the form of p-values and z-scores. A z-score is the measurement of the number of standard deviations the raw score is from the population mean.

2.13 Statistical Analysis

For flow cytometric analyses percentage positive cells, proliferation index, division index or percentage of cells divided were visualised as graphs using GraphPad Prism 5.03 (GraphPad Software Inc.; California, USA). For cell surface markers percentage positive cells were used to create graphs using GraphPad Prism. Various statistical analyses were carried out on the data of three biological replicates or more, including the use of both parametric and non-parametric tests. Student t-tests were performed to analyse parametric data. For non-parametric data, either Wilcoxon matched paired t-test or Mann-Whitney U test were performed depending on whether dependent (paired) or independent samples were the subject of analysis. These statistical tests were performed using GraphPad Prism (GraphPad Software Inc.) and p-values < 0.05 were considered statistically significant.

Chapter 3. In vitro model of IL-6 pre-exposure

3.1 Introduction

Based on the hypothesis outlined in *Chapter 1*, the overarching aim addressed in this chapter is the development of an in vitro system in order to recapitulate the chronic exposure of naïve and memory human CD4⁺ T-cells to a robust, but approximately physiological, level of IL-6 believed to be occurring in vivo in the early stages of RA. The downstream effects of this period of IL-6 pre-exposure on naïve and memory effector phenotypes and transcriptional profiles were then interrogated as outlined in *Chapters 4* and *5* to provide further insight into cytokine mediated priming in immune dysregulation in early RA.

Naïve CD4⁺ T-cells have been shown to be particularly sensitive to IL-6, indicated by the high levels of pSTAT-3, *Chapter 1.3.12*. This increased sensitivity appears to be independent of serum levels of sIL-6R and sgp130 and independent of IL-6R expression. For this reason, the work presented in this chapter aims to assess whether expression of the IL-6 signal transducing subunit gp130 can account for the high sensitivity of this naïve subset compared to the memory subset.

In order to assess the consequences of IL-6 pre-exposure on downstream effector profile and transcriptional profiles, naïve and memory CD4⁺ T-cells will be isolated and subject to a period of pre-exposure to IL-6 before the cytokine is removed from the system and cells are stimulated via the TCR. To ascertain whether effects of IL-6 pre-exposure are more pronounced in naïve CD4⁺ T-cells, as a result of their apparent increased sensitivity, these subpopulations will be isolated and cultured in parallel.

One of the key elements of the in vitro IL-6 pre-exposure model is the isolation of naïve CD4⁺ T-cells and a comparator memory CD4⁺ T-cell population. Naïve and memory CD4⁺ T-cells differ in expression of several T-cell maturity markers which allows their characterisation and isolation for functional applications. Naïve CD4⁺ T-cells express CD45RA whereas their memory counterparts express CD45RO. Memory CD4⁺ T-cells can then be further sub divided into central memory and effector memory by differential expression of CD62L, the lymphocyte homing marker absent on effector memory CD4⁺ T-cells, *Figure 9 Chapter 2.9.4*. A small population of effector T-cells are able to regain the expression of CD45RA and these are termed terminally differentiated cells, these cells are only present in a small proportion of individuals at relatively low levels. The cells that are isolated will impact the interpretation of the data so it is important that isolated populations have high levels of purity.

Another key element to the in vitro model is the IL-6 pre-exposure period. This has several variables which require optimisation including; concentration of IL-6, the presence of sIL-6R and the length of pre-exposure. It is important for the concentration of IL-6 used in the system to be physiologically relevant while still having robust effects on downstream phenotype. It has been described that levels of IL-6 reach up to 184pg/ml in RA sera and 9.46ng/ml in synovium, therefore to ensure physiological relevance a range of IL-6 concentrations will be tested (265, 452, 453). As previously mentioned IL-6 can signal via membrane bound IL-6R or sIL-6R in classical and trans signalling respectively, *Chapter 1.3.2*. The presence of sIL-6R in the culture system enables the possibility of trans signalling, whereas the absence will restrict the in vitro model to predominantly classical signalling. The different signalling pathways of IL-6 may have potential implications on downstream effector phenotype. The inclusion of sIL-6R will encompass both signalling mechanisms, ensuring the system will be saturated and will result in high levels of signalling to ensure any effect of pre-exposure is observed. The length of culture of cells during the pre-exposure period also needs to be carefully determined in order to balance chronicity of exposure, as occurs in early RA, with the viability of cells in the absence of TCR stimulation.

All aspects of the in vitro model need to be carefully optimised in order to balance approximation to the physiological state with experimental reductionism in order for the effect of IL-6 pre-exposure to be determined.

3.2 Aims and Objectives

The work outlined in this chapter aims to establish a robust system for isolating naïve and memory CD4⁺ T-cells from healthy controls and culturing in the presence or absence of IL-6 before its removal from the system followed by stimulation via the TCR to examine the effects of IL-6 pre-exposure on CD4⁺ T-cell effector function. The role of this optimised in vitro model is to mimic the situation in early RA patients where cells are chronically exposed to circulating IL-6. Presence of IL-6 at time of stimulation of CD4⁺ T-cells has been widely studied and for this study, we are interested in the cytokine pre-priming effects of prior exposure to IL-6 on circulating CD4⁺ T-cells. This in vitro system can then be used to investigate the effect of chronic IL-6 stimulation and assess its ability to influence effector phenotype following TCR mediated activation in terms of proliferation, activation and cytokine production, *Chapter 4*.

The specific objectives were to:

- Assess the expression of IL-6 signalling components on CD4⁺ T-cell subsets
- Determine the optimal method of naïve CD4⁺ T-cell isolation.

- Obtain a comparator memory cell fraction for each isolation.
- Assess the optimal culture conditions of naïve CD4⁺ T-cells in terms of culture medium, length of culture and culture with cytokines.
- Examine the phenotypic consequences of pre-exposure of naïve and memory CD4⁺ T-cells to IL-6 prior to TCR mediated activation.
- Determine the optimal method of TCR stimulation.

3.3 Results

3.3.1 Expression of IL-6 receptor components on CD4⁺ T-cell subsets

It was observed in *Chapter 1.3.12* that there is an apparent increase in sensitivity of CD45RA⁺CD62L⁺ naïve CD4⁺ T-cells to IL-6, documented by the strong correlation between serum IL-6 and activated STAT-3 (*Figure 8, Chapter 1. 3.12*). This was not related to IL-6R expression, which was maximal on central memory CD4⁺ T-cells suggesting a mechanism other than IL-6R expression is responsible for increased sensitivity of these cells to IL-6. For this reason, the initial focus was to examine whether this increased sensitivity was due to differences in expression of the gp130 signalling component.

The expression levels of both gp130 and IL-6R on CD4⁺ T-cells of patients from the NEAC were assessed by whole blood staining, the gating strategy for this analyses, employing the setting of gates on internal negative controls, *Methods 2.9.5*, is depicted in *Figure 8, Appendix*. Clinical information of these patients can be seen in *Table 12*. Whole blood was stained with a panel of antibodies to identify CD3⁺CD4⁺ T-cells and expression of CD45RA and CD62L were used to determine true naïve and memory cell sub-populations. Staining of these populations for IL-6R using the IL-6R-PerCPeFluor710, clone 47.797.1F2, reflected expression previously seen in *Figure 8, Chapter 1.3.12*. Representative dot plots show fewer naïve CD4⁺ T-cells positive for IL-6R but a higher number of cells positive for gp130, *Figure 12A and C*. This was consistent amongst 27 donors with the median represented in *Figure 12B and D*. True naïve CD4⁺ T-cells had the lowest levels of IL-6R, with the highest expression on central memory CD4⁺ T-cells, with moderate levels on effector memory CD4⁺ T-cells, *Figure 12A-B*. This pattern could also be seen using a different antibody, IL-6R-CFS, clone 17506 recognising a different epitope of the IL-6R, *Figure 9, Appendix*. In contrast, staining of these populations revealed maximal expression of gp130 on true naïve CD4⁺ T-cells, with reduced levels on central memory and further reduced levels on effector memory cells, *Figure 12C-D*. This increased expression of the IL-6R β signalling subunit provides a possible explanation as to the

apparent increased sensitivity of naïve CD4⁺ T-cells to IL-6 and a potential mechanism for increased levels of trans signalling.

	Rheumatoid Arthritis (n=8)	Other Inflammatory (n=7)	Non-Inflammatory (n=11)
Age (years, mean, SD)	61 (49-73)	48 (26-70)	47 (37-57)
% Female	63	29	72
CRP (g/l; median)	18.5	7	4
ESR (s, median)	40.5	9	2
ACPA+	6	1	1
RF+	6	2	2

Table 12. Clinical characteristics of EAC attendees that donated whole blood for gp130 and IL-6R expression staining. Table showing the clinical characteristics of EAC attendees including age, sex, levels of C-reactive protein (CRP), erythrocyte sedimentation rate (ESR) and anti-citrullinated peptide antibody (ACPA) and rheumatoid factor (RF) status.

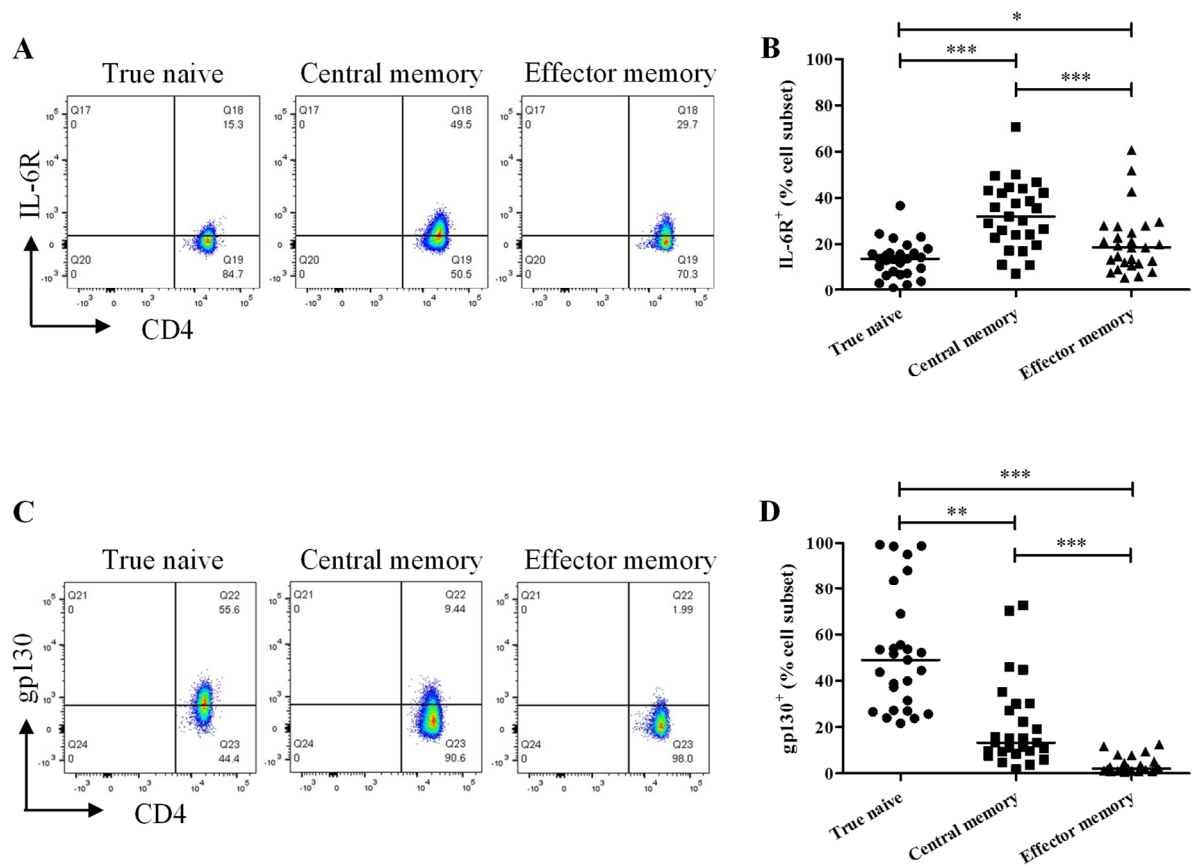


Figure 12. Expression of IL-6R and gp130 on CD4⁺ T-cell subsets of early arthritis patients. Whole blood of early RA patients was stained for flow cytometry with a panel of antibodies to detect cell surface expression of IL-6R (A-B) and gp130 (C-D). CD4⁺ T-cells were identified as CD3⁺CD4⁺ and from this population true naïve cells were identified as CD45RA⁺CD62L⁺, central memory cells identified by CD45RA⁻CD62L⁺ and effector memory cells identified as CD45RA⁻CD62L⁻. Proportions of IL-6R (A) and gp130 (C) positive cells were determined by gating on internal negative controls, with representative dot plots from one donor showing the percentage of cells positive for IL-6R and gp130 (A and C). Data from 27 individual donors were plotted as graphs with horizontal bars representing the median value, significance was determined by non-parametric analysis of variance (Friedman) with Dunn's posthoc pairwise analyses: ** $p < 0.001$, *** $p < 0.0001$, (B and D).

In summary, there are high levels of gp130 on naïve CD4⁺ T-cells compared to memory CD4⁺ T-cells and of the memory subsets, the effector memory cells have the lowest expression of gp130. Alternatively central memory CD4⁺ T-cell express the highest level of IL-6R with true naïve CD4⁺ T-cells having the lowest level of IL-6R. Due to the difference in expression of IL-6 receptor subunits on naïve and memory CD4⁺ T-cells it was decided that future experiments should compare the responses of both naïve and memory CD4⁺ T-cells to IL-6.

3.3.2 Isolation of naïve CD4⁺ T-cells

The initial focus in the optimisation of the in vitro model was to determine a method of naïve CD4⁺ T-cell selection yielding an equivalent population of memory CD4⁺ T-cells. The method

of isolation would need to provide sufficient numbers for use in subsequent experiments but also high purity with as little cell contamination as possible to ensure measured response was a result of naïve or memory CD4⁺ T-cells.

Whereas in *Chapter 3.3.1* identification of naïve cells was aided by the expression of CD62L this was not feasible during cell isolation thus further investigations involve the identification of naïve cells by only CD45RA expression.

Isolation of naïve CD4⁺ T-cells can be achieved by positive selection or enrichment (also called negative selection). Several means of naïve CD4⁺ T-cell isolation were tested in this *Chapter*, including two positive selection and three enrichment techniques, as described in *Chapter 2.4*. The isolation methods are termed as follows:

- PluriBead isolation (positive selection)
- CD45RA MicroBead selection (positive selection)
- EasySep naïve CD4⁺ T-cell isolation kit (enrichment)
- MACS naïve CD4⁺ T-cell isolation kit (enrichment)
- CD45RO MicroBead selection (enrichment)

Each isolation method was carried out on fresh whole blood or LRS cones. Total numbers of naïve CD4⁺ T-cells were determined to give absolute yield and percentage yield was calculated, based on the number of naïve CD4⁺ T-cells in the starting population of PBMC or CD4⁺ T-cells, *Table 13*. To determine the presence of naïve and memory cells in both the final purified naïve population and the remaining fraction immediately following isolation both populations and the starting populations were stained with a naïve purity panel of antibodies for flow cytometry, *Table 4, Chapter 2.9.1*. The gating strategy for purity analyses employs the approach of setting gates on starting population of CD4⁺ T-cells (*Methods, Chapter 2.9.5*), and can be seen in *Figure 2, Appendix*. Here, expression of CD45RA showed clear positive and negative populations which were easily distinguishable on the starting population, gates were set on this population and applied directly to the final populations and remaining populations. In some cases the gates on the final population of naïve CD4⁺ T-cells required adjusting, this is because on a purified population of cells the antibody to antigen ratio may be decreased if not enough antibody is added. In these cases the gates shift to the left as less antibody binds per cell, to correct for this the antibody needs to be optimised for use on purified naïve CD4⁺ T-cells. A representative of the starting populations of PBMCs or CD4⁺ T-cells, purified naïve CD4⁺ T-cell populations and the remaining negative fraction for each isolation can be seen in *Figure 13*.

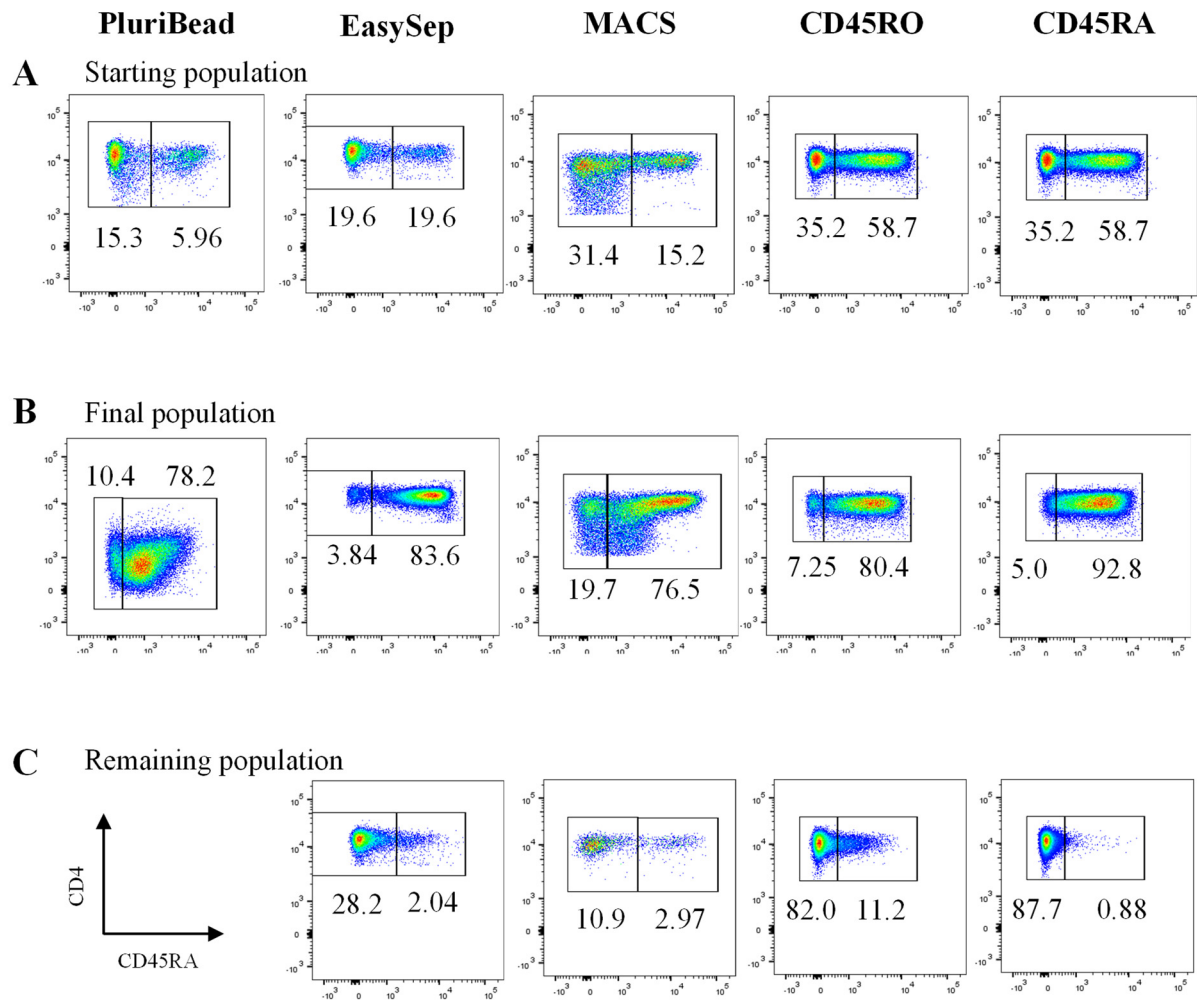


Figure 13. Purity of naïve CD4⁺ T-cells for each isolation method. Naïve CD4⁺ T-cells were isolated from LRS cones or healthy blood using PluriSelect positive selection, Stemcell EasySep naïve CD4⁺ T-cell enrichment, Miltenyi naïve CD4⁺ T-cell MACS enrichment, CD45RO MicroBead isolation or CD45RA MicroBead positive selection. Cells were stained for flow cytometry with a panel of antibodies to detect cell purity (this population was not available for the PluriBead isolation method). Singlets were gated, followed by lymphocytes and CD4⁺ T-cells were identified as CD3⁺CD4⁺. Naïve CD4⁺ T-cells were identified as CD4⁺CD45RA⁺ and memory CD4⁺ T-cells identified as CD4⁺CD45RA⁻, these gates were set on the starting population and then applied to the final and remaining populations with slight adjustments required for the final population. Purity of each population is shown in the flow cytometry plots and was calculated as the percentage of CD45RA⁺ of total alive lymphocytes for the naïve and memory CD4⁺ T-cells from the starting population of PBMC or CD4⁺ T-cells (A), the final population of purified naïve CD4⁺ T-cells (B) and the non-naïve fraction (C). Flow cytometry plots representative of one experiment.

Type of Isolation	Selection Method	Absolute Yield (x10 ⁶)	Percentage Yield	Cell Purity (CD45RA ⁺ % of alive lymphocytes)
Positive selection	PluriBead	1.84	118	78.2
Enrichment	EasySep	6.96	12	83.6
Enrichment	MACS	9.84	99	76.5
Enrichment	CD45RO	8.44	61	80.4
Positive selection	CD45RA	7.04	58	92.8

Table 13. Yield and purity of naïve CD4⁺ T-cell isolations. Percentage yield of naïve CD4⁺ T-cells was calculated by dividing the number of pure CD45RA⁺ cells (absolute yield corrected for purity) by the number of CD45RA⁺ cells expected to isolate from the starting population of PBMC/CD4⁺ T-cells. Purity was calculated as a percentage of CD45RA⁺ of total alive lymphocytes.

Isolation of naïve CD4⁺ T-cells using the PluriBead positive selection protocol (PluriSelect) produced a percentage yield of 118% (*Table 13*) meaning a higher number of cells were isolated than expected based on the number in the starting population of CD4⁺ T-cells. This method also had one of the lowest levels of purity with only 78.2% of lymphocytes being CD45RA⁺, *Figure 13B*. Isolation of naïve CD4⁺ T-cells using CD4⁺ RosetteSep (Stemcell Technologies) to obtain untouched CD4⁺ T-cells followed by CD45RA⁺ MicroBead positive selection (Miltenyi Biotec) gave a good yield of cells with the highest purity, with 58% and 92.8% respectively, and had almost no naïve CD4⁺ T-cells remaining in the negative fraction. Positive selection procedures involve antibody binding to CD45RA, which could have potential implications on downstream phenotype by the activation of signalling pathways in the naïve CD4⁺ T-cells. For this reason, negative enrichment for naïve CD4⁺ T-cells was favoured, as this will result minimal background signalling in the naïve CD4⁺ T-cells, which may have adverse consequences on subsequent experiments.

Isolation of naïve CD4⁺ T-cells by EasySep naïve CD4⁺ T-cell enrichment (Stemcell Technologies) gave an extremely low yield of 12% from the starting PBMC population, *Table 13*. Although this isolation method gave a high purity with 83.6% of cells in the final population being CD45RA⁺ (*Figure 13*), the yield of this method was too low for this isolation method to be a viable option for future experiments. Isolation using MACS naïve CD4⁺ T-cell isolation kit (Miltenyi Biotec) resulted in a yield of 99%, *Table 13*, however the final population of naïve CD4⁺ T-cells had a high number of contaminating memory CD4⁺ T-cells remaining in the purified population meaning a purity of only 76.5%, *Figure 13B*. The naïve CD4⁺ T-cell

isolation method selected for use in subsequent experiments involved enrichment of CD4⁺ T-cells by RosetteSep (Stemcell Technologies) followed by CD45RO MicroBead selection (Miltenyi Biotech). This method, although not the most efficient, gave a high level of purity at 80.4% (*Figure 13B*) and a good yield of 61% (*Table 13*). This method also provides untouched naïve CD4⁺ T-cells, which is beneficial as this will limit any signalling potentially caused by antibody binding in these cells. These data suggest that the purified population is largely of the naïve CD4⁺ T-cell phenotype using this method, with only low levels of memory cell contamination; and importantly, sufficient cell yields for use in subsequent experiments can reliably be obtained.

To summarise, the method of isolation of naïve and memory CD4⁺ T-cells used for the remainder of experiments in this thesis involved enrichment of CD4⁺ T-cells by RosetteSep (Stemcell Technologies) followed by selection of memory CD4⁺ T-cells by CD45RO MicroBeads (Miltenyi Biotech).

3.3.3 Characterising naïve and memory CD4⁺ T-cell phenotype pre-IL-6 exposure

Prior to characterising the effects of IL-6 exposure on naïve and memory CD4⁺ T-cells, it was necessary to validate the phenotype of these isolated cells at baseline. The phenotype of both naïve and memory CD4⁺ T-cells was analysed by staining with a phenotype panel of antibodies for flow cytometry immediately following isolation from LRS cones. This panel of antibodies contained various cell surface markers to assess activation CD25, CD27, CD28, and IL-6 signalling components IL-6R and gp130. Both CD27 and CD28 are known T-cell co-stimulatory molecules required for T-cell immunity. Another activation marker is CD25, the IL-2R α chain, which is found on activated T-cells but is also a marker of Treg cells. The gating strategy for this analysis, employing the approach of setting gates on starting population of CD4⁺ T-cells (*Chapter 2.9.5*), is depicted in *Figure 3, Appendix*.

Representative plots show that there are more cells negative for both CD27 and gp130 expression and more cells positive for IL-6R expression in the memory CD4⁺ T-cells compared to naïve CD4⁺ T-cells, *Figure 14A-B*. Expression of CD28 and CD27 is high on both naïve and memory CD4⁺ T-cells, although in the case of CD27 expression is higher on naïve CD4⁺ T-cells than memory CD4⁺ T-cells, *Figure 14C*. In contrast, CD25 expression is much lower with similar levels between naïve and memory CD4⁺ T-cells. Levels of IL-6R are around 40% for memory cells but there is significantly lower expression on naïve CD4⁺ T-cells, of around 20%, consistent with *Figure 12* , *Chapter 3.3.1*, showing expression of IL-6R is higher on memory CD4⁺ T-cells, particularly the central memory subset of CD4⁺ T-cells. In contrast, the previously assumed ubiquitously expressed gp130 receptor was found to be

substantially higher on naïve CD4⁺ T-cells, with around 90% positive compared to around 50% positive for memory CD4⁺ T-cells, *Figure 14*. This reflects *Figure 12, Chapter 3.3.1*, which shows increased expression of gp130 in naïve CD4⁺ T-cells compared to central and effector memory CD4⁺ T-cells. However, levels of expression are much higher in cells which were analysed following naïve and memory CD4⁺ T-cell isolation, *Figure 14*, compared to whole blood staining, *Figure 12*, which may be a consequence of the isolation procedure.

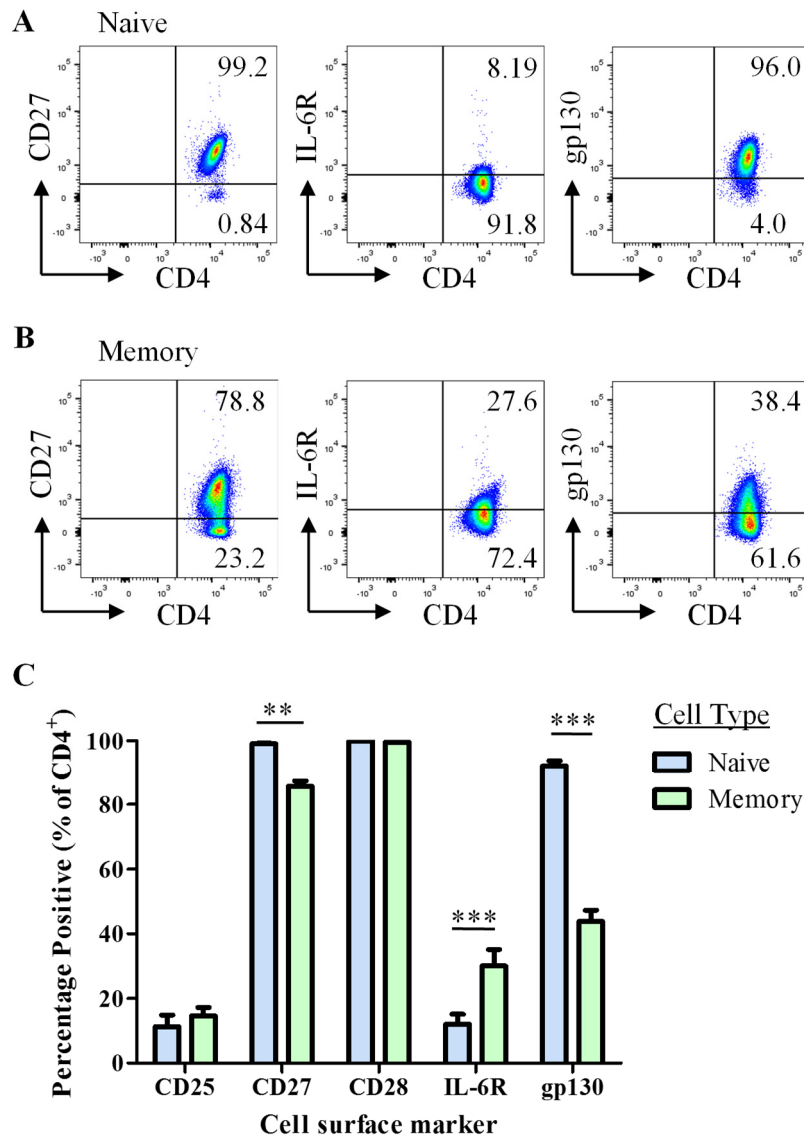


Figure 14. Naïve and memory CD4⁺ T-cell basal cell surface marker expression. Freshly isolated naïve and memory CD4⁺ T-cells were stained for phenotype cell surface markers for flow cytometry. Cells were gated as singlets, lymphocytes and CD4⁺ T-cells were identified as CD3⁺CD4⁺, naïve CD4⁺ T-cells were identified as CD4⁺CD45RA⁺ and memory CD4⁺ T-cells identified as CD4⁺CD45RA⁻. Gates were set on starting populations of CD4⁺ T-cells and applied to naïve and memory CD4⁺ T-cells. Expression of CD25, CD27, CD28, IL-6R and gp130 were measured on naïve CD4⁺ T-cells (**A**) and memory CD4⁺ T-cells (**B**), with representative plots for one donor shown for CD27, IL-6R and gp130. Values from 9 biological replicates were plotted on a graph with bars showing the median and error bars showing SEM (**C**). Significance was determined by 2 way ANOVA with Bonferroni post hoc pairwise analyses; **p<0.01, *** p<0.001.

In summary, naïve CD4⁺ T-cells show significantly lower expression of IL-6R and higher expression of gp130, validating the phenotype of the cells seen in whole blood staining, as in *Chapter 3.2.1*. As expected there was no difference in expression of CD25 between naïve or

memory CD4⁺ T-cells. Furthermore, lower expression of CD27 is seen on memory CD4⁺ T-cells, in keeping with published literature (454).

3.3.4 Culture of naïve and memory CD4⁺ T-cells

Following naïve and memory CD4⁺ T-cell characterisation the next aim was to develop an in vitro culture system in which the effects of IL-6 pre-exposure could be assessed. There were a number of factors to consider when determining the optimal cell culture conditions, including the type of medium used, the length of culture and the addition of cytokines and whether these cytokines are refreshed during culture. These factors have the potential to influence cell viability and response to TCR stimulation.

An important factor to consider in the culture of naïve and memory CD4⁺ T-cells was the type of culture media used. RF10 is a medium used for the culture of cells, which typically contains around 10% FCS, a widely used serum-supplement for in vitro cell culture as it contains growth factors and protein components to assist cell survival and growth. Although this can greatly increase cell survival there are a number of undefined proteins present which can lead to stimulation of cells and activation of signalling pathways. TexMACS (Miltenyi Biotec) is a serum free media designed specifically for T-cells, which contains only a small number of human serum proteins, thereby limiting the amount of signalling through the naïve or memory CD4⁺ T-cell. The effect of media selection upon viability and phenotype of cultured naïve CD4⁺ T-cells was assessed after 10 days culture. Naïve CD4⁺ T-cells, isolated from LRS cones, were cultured in duplicate in RF10 or TexMACS. After 10 days, phenotype and viability were assessed by flow cytometry, gates were set on the starting population of CD4⁺ T-cells (*Chapter 2.9.5*) and for viability analysis clear populations allowed for placement of gates, gating can be seen in *Figures 3 and 5, Appendix*. There appeared to be a decrease in the level of expression of CD27 and gp130 but an increase in expression of IL-6R as a result of 10 days of culture compared to naïve CD4⁺ T-cells at baseline, *Figure 15*. Despite this, there was no large differences in cell surface markers after 10 days of culture in RF10 compared to TexMACS *Figure 15A*. No clear differences could be seen in terms of cell viability with RF10 giving a viability of around 21% compared to a viability of 29% following culture in TexMACS for 10 days, *Figure 15B*. The low level of viability seen may reflect the long culture period of 10 days, which appears to be too long for cells to remain viable, however this is only one donor so may be a reflection of donor variation.

In light of these findings, future experiments using the optimised in vitro model would use TexMACS as the culture media, unless otherwise stated, to prevent the possibility of downstream signalling events, which may affect subsequent read out of future experiments.

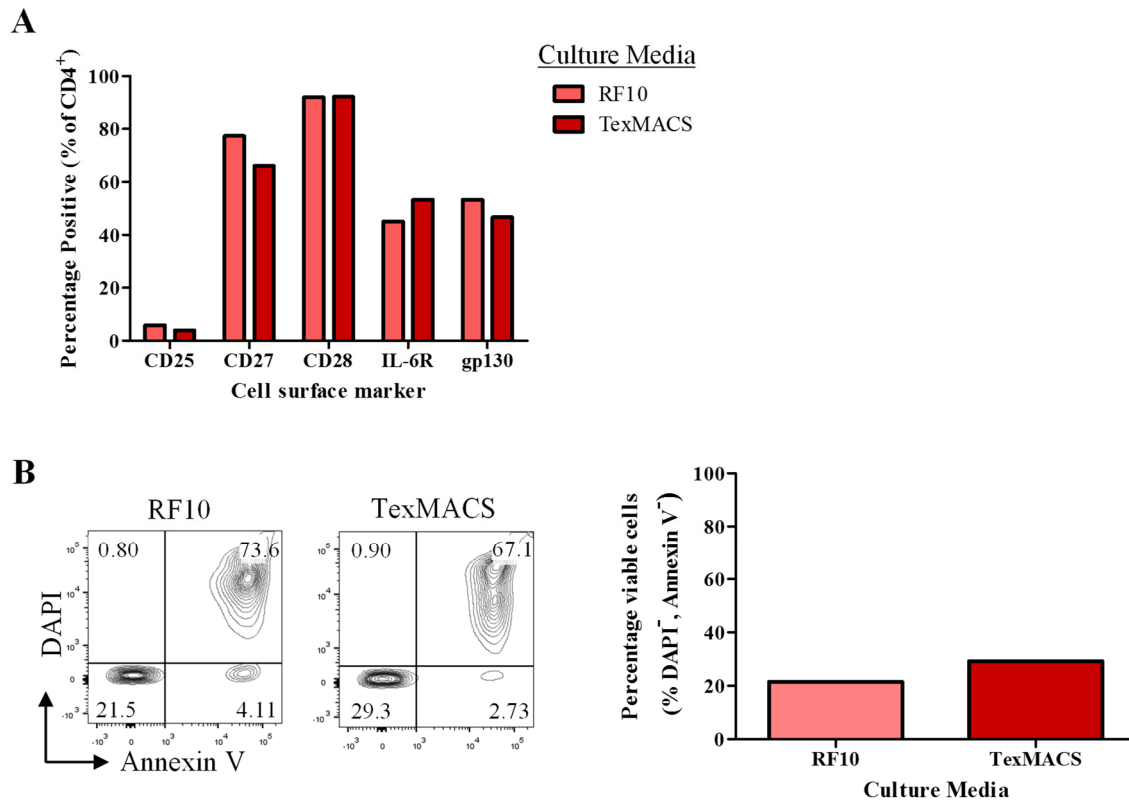


Figure 15. Comparison of cell culture media on naïve CD4⁺ T-cell phenotype and viability. Freshly isolated naïve CD4⁺ T-cells were cultured for 10 days in RF10 or TexMACS. After 10 days cells were stained for cell surface markers for phenotype analysis (**A**) or with Annexin V and DAPI for viability analysis (**B**). For phenotype analysis cells were gated as singlets, lymphocytes and CD4⁺ T-cells were identified as CD3⁺CD4⁺, naïve CD4⁺ T-cells were identified as CD4⁺CD45RA⁺ and memory CD4⁺ T-cells identified as CD4⁺CD45RA⁻. Gates were set on starting populations of CD4⁺ T-cells and applied to naïve CD4⁺ T-cells. Expression of CD25, CD27, CD28, IL-6R and gp130 were measured (**A**). For viability cells were gated as DAPI⁺Annexin V⁻ (**B**). N=1.

3.3.5 Characterising naïve and memory CD4⁺ T-cell phenotype post-IL-6 exposure

Next, the phenotype and viability of naïve CD4⁺ T-cells was assessed following exposure to IL-6 and or equimolar sIL-6R in order to determine the optimal conditions for culture with cytokine.

The phenotype of naïve CD4⁺ T-cells, isolated from LRS cones, was assessed following 3 days of culture in the presence of 50ng/ml IL-6 with equimolar sIL-6R, included to maximise signalling via both the classical and trans mechanisms. Expression of CD25, CD27 and CD28 on naïve CD4⁺ T-cells following 3 days of culture remain at similar levels as detected in *Chapter 3.3.3* in the starting population of naïve CD4⁺ T-cells in both untreated and IL-6 exposed cells, *Figure 16A*. There was also no change in these cell surface markers when

cultured alone compared to culture with IL-6 and sIL-6R (*Figure 16A*) indicating the cells remain in their naïve, phenotypically inactivated state during culture with IL-6.

By contrast, expression of both IL-6R and gp130 dramatically decreases following 3 days of culture in the presence of 50ng IL-6 and equimolar sIL-6R, *Figure 16A*, compared to when cultured in media alone where expression levels remain similar to that seen immediately following isolation, *Figure 14, Chapter 3.3.3*. There is no difference in the expression of these markers when cultured with IL-6 alone compared to when cultured with IL-6 and sIL-6R, *Figure 10, Appendix*. However, this observation is only representative of one experiment.

Again, there was a low level of viability following 10 days culture with a small decrease in viability seen between cells cultured alone compared to cells cultured with IL-6 alone with percentage viable cells 29% and 17%, respectively. There is no difference in viability between cells cultured in IL-6 alone compared to cells cultured with IL-6 + sIL-6R with both conditions having around 16-17% viability after 10 days, *Figure 16B*.

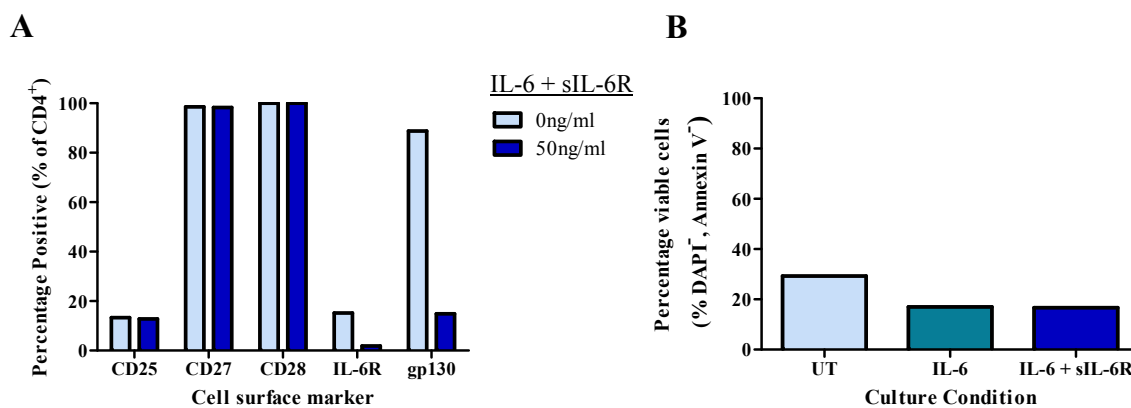


Figure 16. Surface marker expression of untreated and IL-6 treated naïve CD4⁺ T-cells following 3-day culture. Freshly isolated naïve CD4⁺ T-cells were cultured for 10 days in TexMACS alone or TexMACS with 50ng/ml IL-6 and equimolar sIL-6R. After 3 days cells were stained for cell surface markers for phenotype analysis (A) or with Annexin V and DAPI for viability analysis (B). Viable cells were gated as DAPI⁺Annexin V⁻. Cells were gated as singlets, followed by lymphocytes and CD4⁺ T-cells were identified as CD3⁺CD4⁺, naïve CD4⁺ T-cells were identified as CD4⁺CD45RA⁺ and memory CD4⁺ T-cells identified as CD4⁺CD45RA⁻. N=1.

Next, the response of naïve CD4⁺ T-cells to refreshment of cytokines during culture was evaluated. Naïve CD4⁺ T-cells were cultured for 10 days in duplicate in RF10 alone or with final concentration of 50ng/ml IL-6 and equimolar sIL-6R. For one well of each condition culture medium was refreshed on days 3 and 6, with the same final concentrations of IL-6 and sIL-6R added as on Day 0. As seen in *Figure 16* there was no change in CD25, CD27 or CD28

expression with culture with IL-6 and no difference was seen with refreshing cytokines compared to unrefreshed cells, *Figure 17A*. In contrast to the previous experiments, the viability of cells was much higher for this donor, around 60-70%, *Figure 17B*. However, addition of IL-6 and the refreshment of cytokines also had no impact on cell viability after 10 days culture, with a difference of only 3-8% in viability with cytokine refreshment, *Figure 17B*.

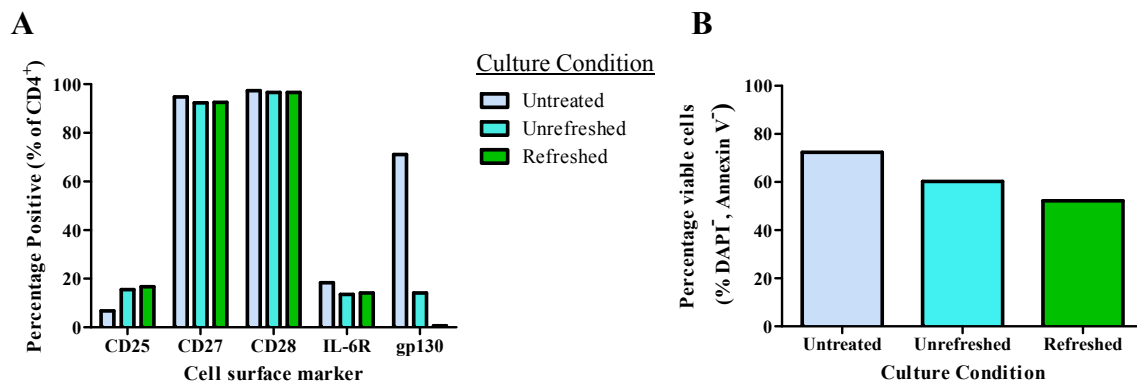


Figure 17. Comparison of refreshing culture media with IL-6 and sIL-6R on naïve CD4⁺ T-cell phenotype and viability. Freshly isolated naïve CD4⁺ T-cells were cultured for 10 days in RF10 alone or RF10 with 50ng/ml IL-6 and equimolar sIL-6R. Conditions were carried out in duplicate and to one of each conditions 50ng/ml IL-6 or 50ng/ml IL-6 and equimolar sIL-6R were added to the culture on day 3 and day 6. After 10 days cells were stained for cell surface markers for phenotype analysis (**A**) or stained with Annexin V and DAPI for viability analysis (**B**). Viable cells were gated as DAPI⁺Annexin V⁻. Cells were gated as singlets, followed by lymphocytes and CD4⁺ T-cells were identified as CD3⁺CD4⁺. N=1.

Further to this, the effect of culture with varying concentrations of IL-6 and equimolar concentrations of sIL-6R on naïve CD4⁺ T-cell viability and phenotype was also measured over 3 days, to further investigate the downregulation of IL-6R and gp130. Naïve CD4⁺ T-cells were cultured for 1-3 days either alone or exposed to IL-6 at final concentrations of 0.1ng/ml, 0.5ng/ml, 5ng/ml and 50ng/ml all with equimolar concentrations of sIL-6R. Levels of cell surface markers were assessed by flow cytometry on each day throughout the culture. There was no effect of differing concentrations of IL-6 on any of the surface markers, CD25, CD27 or CD28, (*Figure 11, Appendix*). However, levels of both IL-6R and gp130 show a dose dependent decrease with increasing concentrations of IL-6 and equimolar sIL-6R, *Figure 18*. This decrease in expression is most striking for gp130 as expression of this molecule remains at a high level of over 80% throughout the culture in untreated naïve CD4⁺ T-cells. The biggest observed difference occurred between 0.5ng/ml and 5ng/ml at each time point during the culture. Furthermore, the expression of these markers was decreased from baseline levels following only 24 hours exposure to IL-6 and equimolar sIL-6R and this reduced expression

remained throughout days 2-3 of culture, *Figure 18*. This confirms the observations that culture with IL-6 reduces expression of gp130, indicating that it is an early event following exposure.

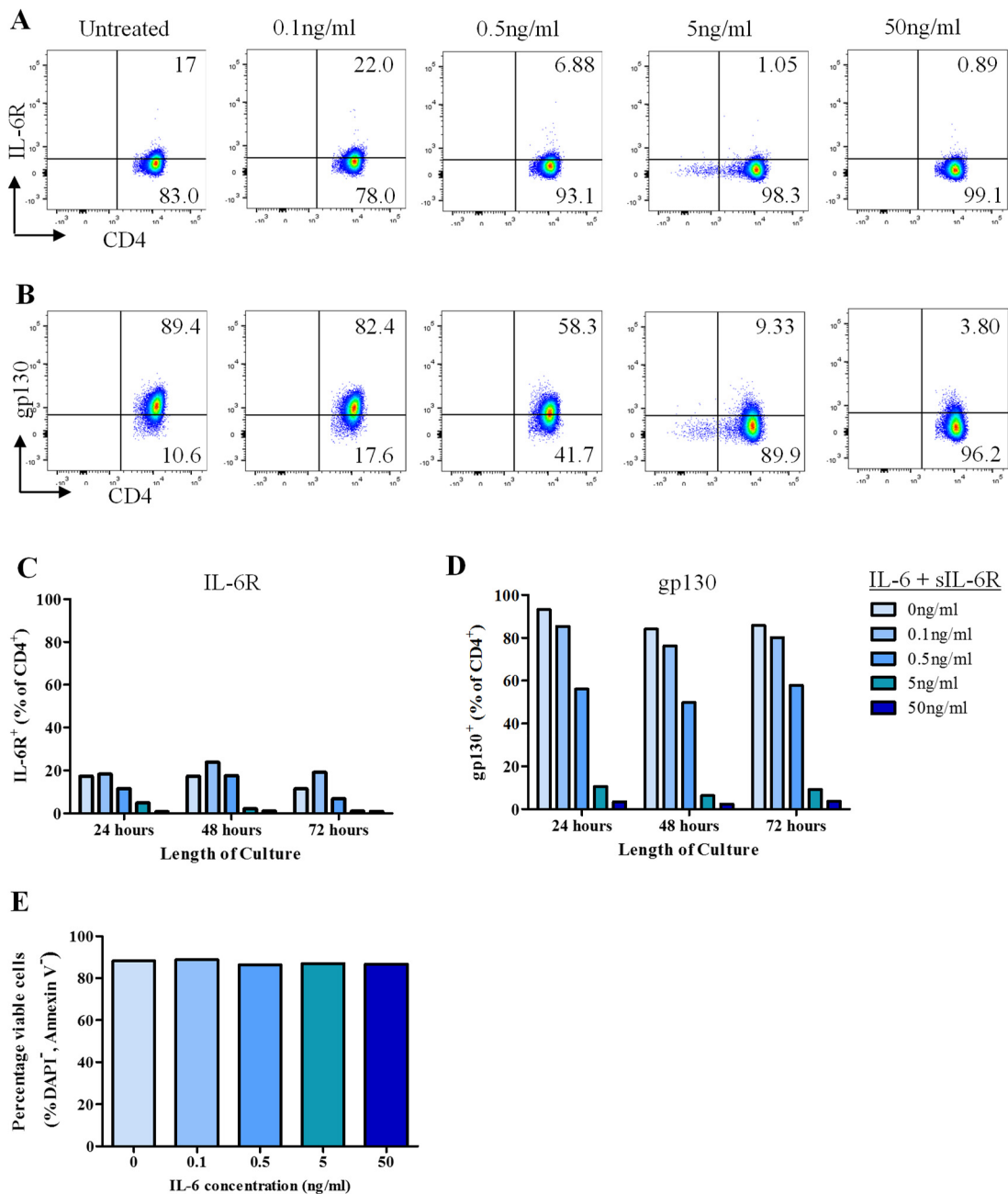


Figure 18. Effect of culture with different concentrations of IL-6 and sIL-6R on naïve CD4⁺ T-cell viability and phenotype. Freshly isolated naïve CD4⁺ T-cells were cultured for 3 days in TexMACS alone, with 0.1ng/ml IL-6, 0.5ng/ml IL-6, 5ng/ml IL-6 or 50ng/ml IL-6, all with equimolar sIL-6R. On days 1-3 cells were stained for cell surface markers for flow cytometry, representative plots shown for IL-6R (A) and gp130 (B) at day 3. IL-6R (C) and gp130 (D) expression was similar over 3 days of culture. On day 3 cells were stained with Annexin V and DAPI for viability analysis (E). Viable cells were gated as DAPI⁺. Cells were gated as singlets, followed by lymphocytes and CD4⁺ T-cells were identified as CD3⁺CD4⁺. N=1.

Despite the changes in cell surface marker expression at 3 days of culture there was no production of cytokines, IFN- γ , IL-10, IL-4 or IL-5, either when cultured alone or when cultured with varying concentrations of IL-6 with equimolar sIL-6R with supernatants having below the level of detection for each cytokine (*Figure 12, Appendix*).

As a result of these data subsequent experiments involved culture of naïve and memory CD4⁺ T-cells with varying concentrations of IL-6 and equimolar concentrations of sIL-6R without further refreshment of cytokines.

3.3.6 Optimal length of naïve CD4⁺ T-cell culture

After determining, the optimal conditions for the culture of naïve CD4⁺ T-cells in TexMACS with IL-6 and equimolar sIL-6R the next step was to determine the optimal length of culture. During in vitro cell culture viability decreases, especially in the absence of TCR stimulation, with the potential for decreased viability due to culture in serum free media. The maximal length of culture over which naïve CD4⁺ T-cell viability could be maintained in serum free media prior to TCR stimulation was determined by assessing viability of naïve CD4⁺ T-cells over a period of 6 days using Annexin V and DAPI staining. Naïve CD4⁺ T-cells, isolated from LRS cones, were cultured in TexMACS alone or TexMACS with final concentration of 50ng/ml IL-6 and equimolar concentration of sIL-6R, 90ng/ml. Phenotype analysis showed that the level of expression of cell surface markers remained unchanged throughout the 6 days of culture, *Figure 19A*, although gp130 appears to increase throughout the culture. There was no difference in viability between the untreated or IL-6 treated conditions at each time point *Figure 13, Appendix*. During the first three days of culture it can be seen that cells are over 90% viable, which rapidly decreases to less than 40% at day 4, *Figure 19B*. This experiment reflects *Figures 15 and 16* showing low viability, less than 10% after 6 days of culture, *Figure 19B*.

Consequently, it was determined that subsequent experiments should involve pre-exposure to IL-6 for no longer than 3 days, balancing chronicity of IL-6 exposure with cell viability.

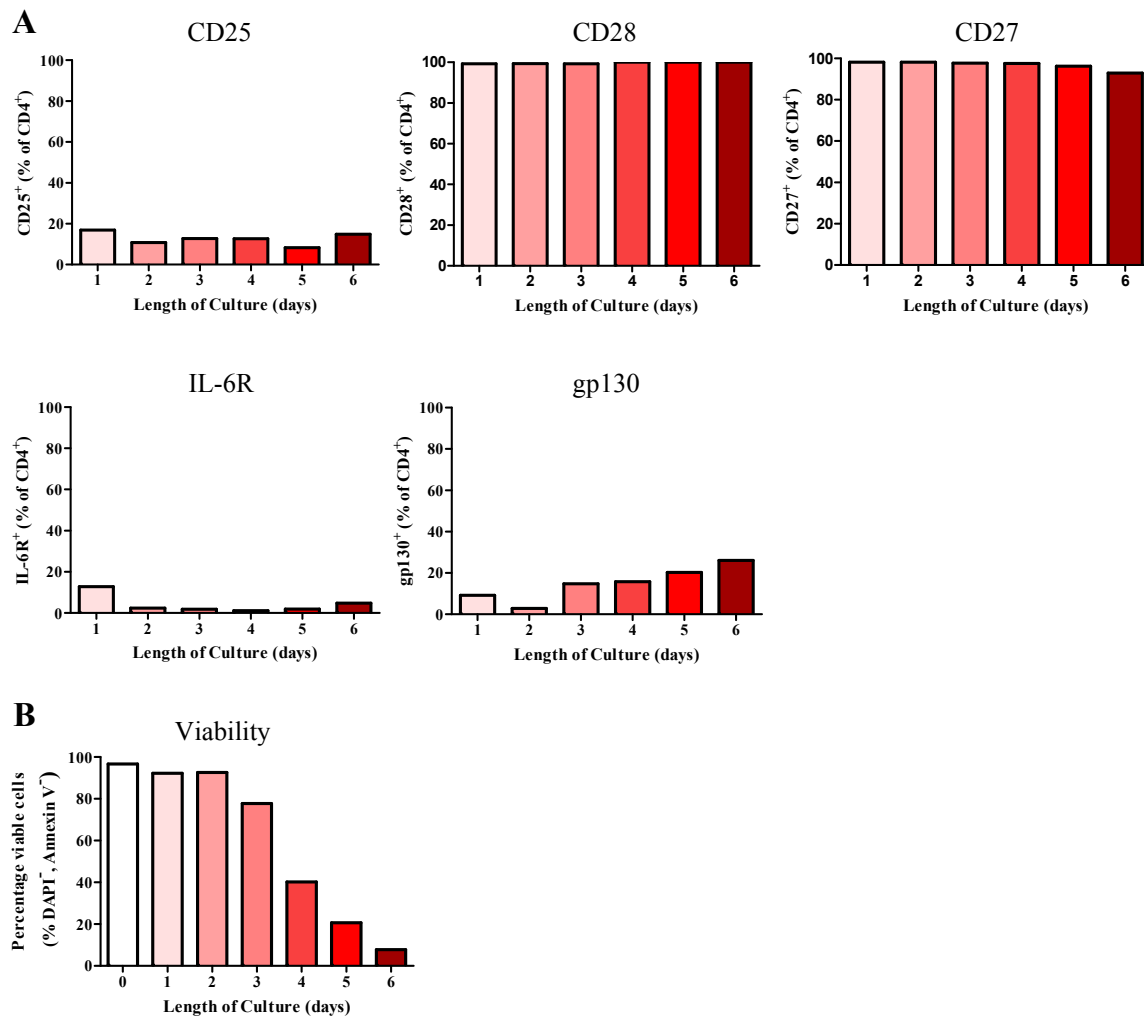


Figure 19. Effect of length of culture with IL-6 and sIL-6R on naïve CD4⁺ T-cell phenotype and viability. Freshly isolated naïve CD4⁺ T-cells were cultured for 1 to 6 days in TexMACS with 50ng/ml IL-6 and equimolar sIL-6R. (A) On days 1-6 cells were stained for cell surface markers for phenotype analysis (A) or stained for Annexin V and DAPI for viability analysis (B). Viable cells were gated as DAPI⁻ Annexin V⁻. Cells were gated as singlets, followed by lymphocytes and CD4⁺ T-cells were identified as CD3⁺CD4⁺. N= 1.

3.3.7 Stimulation of naïve CD4⁺ T-cells

Following naïve and memory CD4⁺ T-cell isolation and culture with varying concentrations of IL-6 and equimolar sIL-6R cells were washed thoroughly prior to TCR stimulation in order to remove all cytokines from the culture media so that no IL-6 will be present at time of stimulation. The stimulation of naïve and memory CD4⁺ T-cells was optimised in terms of anti-CD3 and anti-CD28 concentrations and length of stimulation. This approach carries the potential advantage of allowing the strength of stimulus to be controlled in a manner not possible in an MLR. The aim of the optimised concentration of anti-CD3 and anti-CD28 is to induce sub-optimal levels of proliferation so that any difference with IL-6 pre-exposure will be

more likely to be observed. These optimised conditions will be applied in *Chapter 4* to look in detail at the effect of IL-6 pre-exposure on effector function.

Firstly, the proliferation of naïve and memory CD4⁺ T-cells following IL-6 pre-exposure and subsequent TCR stimulation by anti-CD3 and anti-CD28 followed by tritiated thymidine incorporation assay was assessed. Freshly isolated naïve CD4⁺ T-cells, isolated from LRS cones, were cultured in the absence or presence of IL-6 and sIL-6R, at 0.1ng/ml IL-6 or 50ng/ml IL-6 each in the presence of equimolar sIL-6R for 3 days. Cells were harvested and washed to remove any cytokines present in the media and cultured in the presence of varying concentrations of anti-CD3 and anti-CD28 stimulus, for 6 days. After 6 days tritiated thymidine incorporation assays were carried out, each condition was carried out in triplicate with the median represented in *Figure 20*, this shows that higher concentrations of anti-CD3 and anti-CD28 produce higher levels of proliferation. When stimulated with 1µg/ml anti-CD3 and 0.1-1µg/ml anti-CD28 there are only very low levels of proliferation whereas proliferation was much higher when using 10µg/ml anti-CD28, specifically with 1µg/ml anti-CD3 and 10µg/ml anti-CD28 stimulation concentrations pre-exposure to IL-6 causes an increase in proliferation, *Figure 20B*.

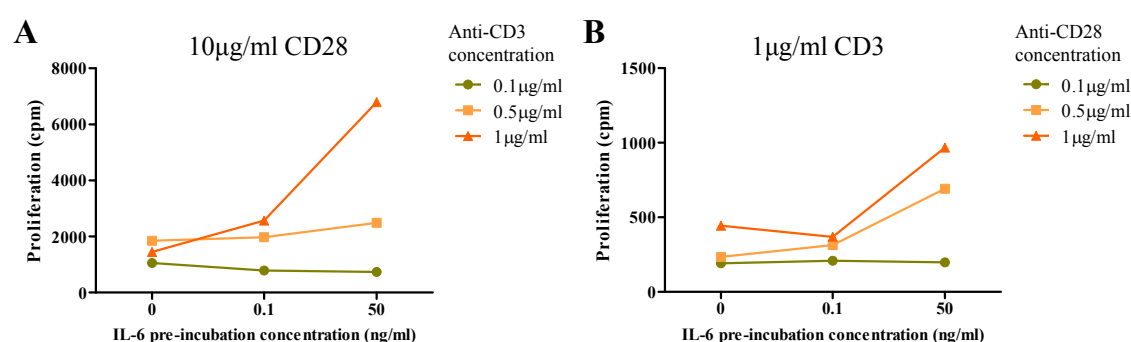


Figure 20. Proliferation of naïve CD4⁺ T-cells following IL-6 exposure and stimulation with anti-CD3 and anti-CD28 and tritiated thymidine incorporation assay. Freshly isolated naïve CD4⁺ T-cells were cultured for 3 days in TexMACS alone, TexMACS with 0.1ng/ml IL-6 or TexMACS with 50ng/ml IL-6 both with equimolar sIL-6R. After 3 days cells were cultured with varying concentrations of anti-CD3 and 10µg/ml anti-CD28 (**A**) or varying concentrations of CD28 with 1µg/ml CD3 (**B**) for a further 6 days. Proliferation was then assessed by tritiated thymidine incorporation assay, each condition was carried out in triplicate with median for each condition represented. Graph shows one experiment with 3 technical replicates.

Next, tritiated thymidine incorporation was measured from naïve CD4⁺ T-cells stimulated for 3, 6 or 9 days with 1µg/ml anti-CD3 and 10µg/ml anti-CD28 to assess the optimal length of stimulation time. Each condition was carried out in triplicate with median for each condition represented in *Figure 21*. This concentration of anti-CD3 and anti-CD28 was chosen due to its ability to induce optimal induction of proliferation in the previous experiment, resulting in a dynamic range of response seen according to pre-exposure to IL-6 and sIL-6R. There is only a weak level of proliferative response of less than 1×10^5 cpm after only 3 days of stimulation, indicating the stimulation time is too short to induce good levels of proliferation, *Figure 21*. Proliferation increases to around 3×10^5 cpm after 6 days stimulation and further increased levels of proliferation can be seen after 9 days stimulation with a proliferative response of just over 4×10^5 cpm.

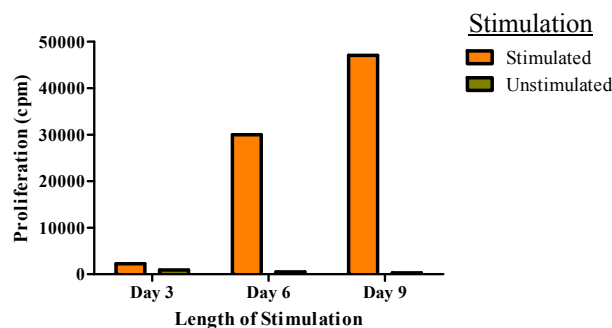


Figure 21. Length of naïve CD4⁺ T-cell stimulation following IL-6 exposure using anti-CD3 and anti-CD28 and tritiated thymidine incorporation assay. Freshly isolated naïve CD4⁺ T-cells were cultured for 3 days in TexMACS 0.5ng/ml IL-6 and equimolar sIL-6R. After 3 days cells were washed and cultured with 1µg/ml anti-CD3 and 10µg/ml anti-CD28 for 3, 6 or 9 days. Proliferation was then assessed by tritiated thymidine incorporation assay, each condition was carried out in triplicate with median for each condition represented. Graph shows one experiment with 3 technical replicates.

Finally, the effect of stimulation with anti-CD3 and anti-CD28 was assessed by CFSE proliferation assay, as a potentially more sensitive system than the tritiated thymidine incorporation assay. Cells were labelled with 0.5µM CFSE, prior to 3-day culture in media alone, with 0.1ng/ml IL-6, 0.5ng/ml IL-6 or 50ng/ml IL-6 all with equimolar sIL-6R. Cells were harvested and washed to remove any cytokines present in the media and cells cultured for a further 3 or 6 days with 1µg/ml anti-CD3 and 5µg/ml anti-CD28 (high stimulus) or 0.5µg/ml anti-CD3 and 1µg/ml anti-CD28 (low stimulus). The two anti-CD3 and anti-CD28 concentrations were chosen to probe whether changes could be seen even when given a strong stimulatory signal; it was possible that this stimulation may cause maximal levels of

proliferation masking any differences caused by IL-6 pre-exposure. Similar to the tritiated thymidine incorporation assay, 3 days of stimulation with anti-CD3 and anti-CD28 results in only minimal levels of proliferation with little difference between increasing concentrations of IL-6, *Figure 22*. In contrast, stimulation of cells for 6 days produced higher levels of proliferation, although as this was carried out with a sub-optimal concentration this level was fairly low, however there is a clear increase with increasing concentrations of IL-6 pre-exposure, with the exception of 0.5ng/ml IL-6 with the high stimulus, *Figure 22B*.

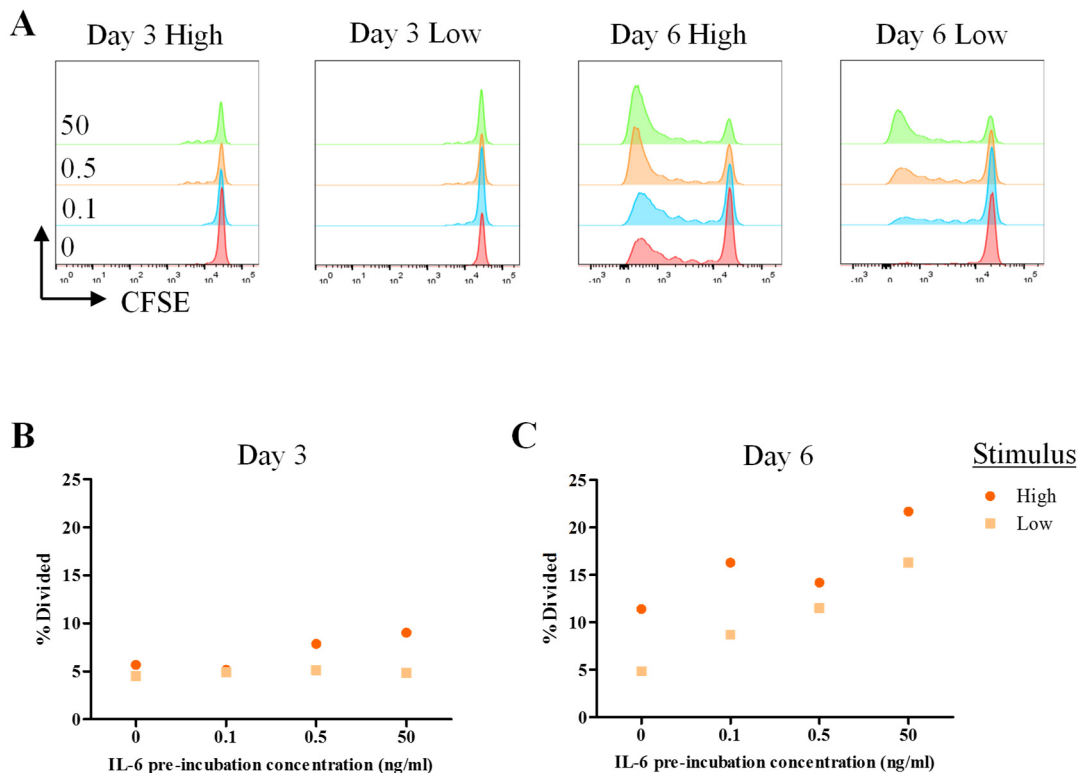


Figure 22. Proliferation of CFSE labelled naïve CD4⁺ T-cells following IL-6 exposure and stimulation with anti-CD3 and anti-CD28. Freshly isolated naïve CD4⁺ T-cells were labelled with 0.5μM CFSE and cultured for 3 days in TexMACS alone, with 0.1ng/ml IL-6, with 0.5ng/ml IL-6 or with 50ng/ml IL-6, all with equimolar sIL-6R. After 3 days cells were cultured with 1μg/ml anti-CD3 and 5μg/ml anti-CD28 (high) or 0.5μg/ml anti-CD3 and 1μg/ml anti-CD28 (low) for a further 3 days or 6 days. Proliferation was then assessed by flow cytometry with representative CFSE histograms (**A**). Proliferation at day 3 (**B**) and day 6 (**C**) were plotted as graphs. Cells were gated as singlets, followed by lymphocytes and proliferating cells were identified as DAPI⁻. Proliferation was calculated using the FlowJo proliferation tool with percentage divided indicating the precursor frequency of dividing cells. N=1.

There was no clear difference between proliferation with the high and low stimulus at day 3, however, at day 6 the high stimulus induced higher levels of proliferation though a better dose-response was seen more clearly with the low stimulus. Thus, a stimulation time of 6 days appears to result in the best level of proliferation when using anti-CD3 and anti-CD28 at either

a high or a low concentration and the CFSE proliferation assay provides good indication of proliferative response of naïve CD4⁺ T-cells.

3.4 Discussion

In this chapter, I set out to establish an in vitro model in which to mimic the chronic pre-exposure of naïve CD4⁺ T-cells to IL-6 prior to TCR mediated stimulation in order to assess the consequences on T-cell effector function. The initial focus was to optimise a method of naïve CD4⁺ T-cell isolation to provide both high yield and high purity. Optimisation experiments determined the length of culture of naïve and memory CD4⁺ T-cells in the absence or presence of varying concentrations of IL-6 and sIL-6R was 3 days. Stimulation of cells was optimised to provide a sensitive assay to reveal any differences in proliferation that may occur between conditions. This involves stimulation with anti-CD3 and anti-CD28 antibodies at varying concentrations for 6 days, using CFSE to measure levels of proliferation.

3.4.1 Expression of IL-6 signalling receptors

In *Chapter 1.3.12* it was seen that naïve CD4⁺ T-cells have a strong correlation between serum IL-6 levels and constitutive intracellular pSTAT-3 indicating these cells are particularly sensitive to circulating IL-6. In this Chapter, it was assessed whether the increased sensitivity of these cells was due to differential expression of the IL-6R responsible for signal transduction.

In this Chapter, the observation shown in previous work, *Figure 8, Chapter 1.3.12* (Amy Anderson, Newcastle University, unpublished data), of higher expression of IL-6R on central memory CD4⁺ T-cells compared to naïve CD4⁺ T-cells, was confirmed. In contrast, naïve CD4⁺ T-cells from EAC patients have higher expression of gp130 compared to both central memory and effector memory CD4⁺ T-cells.

The finding of low expression of IL-6R and high expression of gp130 was also confirmed in naïve CD4⁺ T-cells isolated from healthy donors in *Figure 14*, suggesting this is not a disease specific effect. However, expression of gp130 is much higher on both naïve and memory CD4⁺ T-cells following isolation compared to levels seen in whole blood staining with levels reaching around 95% on freshly isolated naïve CD4⁺ T-cells compared to 50% on naïve CD4⁺ T-cells from whole blood, *Figure 12, Chapter 3.3.1*. This may be due to the sensitivity of this molecule to the cell isolation procedure causing its upregulation, however, the trend still reflects the levels of expression seen with whole blood staining, confirming this observation in healthy controls.

In the published literature gp130 is widely described as ubiquitously expressed across cell types and organs (227). However, it has been described that vascular smooth muscle cells do not constitutively express gp130 but both mRNA and protein can be upregulated upon stimulation

by IL-6 (231). It has further been reported there is differential expression of gp130 according to cell type (455). For example, monocytes express high levels of both gp130 and IL-6R compared to NK cells, which lack both molecules. B-cells have low levels of gp130 but high IL-6R whereas CD4⁺ and CD8⁺ T-cells have variable expression of both markers.

Several studies have now shown that expression of gp130 and IL-6R varies depending upon anatomical location (233, 456). In the case of monocytes, expression of gp130 is reduced in SF in a MAPK dependent manner compared to peripheral blood (233). Similarly, expression of the IL-6R on CD4⁺ T-cells has been shown to be significantly lower in SF and ST compared to peripheral blood of RA patients (456), whereas expression of gp130 was only significantly lower on SF cells compared to peripheral blood. IL-6 has been shown to be present in high concentrations in the ST suggesting there is regulation of receptor expression in the inflamed microenvironment. Our data further suggests another level of regulation dependent upon the maturity phenotype of cells in the periphery.

Expression levels of IL-6R and gp130 were decreased following exposure to IL-6 in a dose-dependent manner, consistent with the literature which suggest IL-6 signalling downregulates IL-6R and gp130 expression and hence the cells' ability to signal via this mechanism (288, 368, 455). This is due to the internalisation of the receptor complex and the synthesis of new receptors is required before further signalling (288). Levels of gp130 and IL-6R remain low until 48 hours following exposure to IL-6 possibly due to the high availability of IL-6 and levels begin to gradually increase with further culture possibly because of decreased IL-6 availability.

The biggest observed difference in both IL-6R and gp130 expression when cultured with IL-6 and equimolar sIL-6R occurs between 0.5ng/ml and 5ng/ml, *Figure 18*. The concentration of 0.5ng/ml IL-6 was chosen as optimal for future experiments, as this was deemed to be at the higher level of the physiological range, however, still result in consequences upon effector function, *Chapter 4.3.8*. There was a small reduction in IL-6R and gp130 expression at this concentration but levels were still comparable to ex vivo levels of expression whereas at 5ng/ml IL-6 levels receptor expression levels were almost completely lost. This is reassuring as it suggests the optimised in vitro model is not creating a completely irrelevant environment.

To summarise, there is a high level of expression of gp130 on naïve CD4⁺ T-cells compared to memory CD4⁺ T-cells. The high expression of gp130 on naïve CD4⁺ T-cells compared to memory CD4⁺ T-cells suggests a potential mechanism of increased sensitivity of these cells to IL-6 signalling and suggests a role for this cell type in mediating the effects of IL-6 pre-exposure.

3.4.2 Defining naïve and memory CD4⁺ T-cells for functional experiments

Isolation of naïve and memory CD4⁺ T-cells involved the use of CD45RO for the positive selection of memory CD4⁺ T-cells. In contrast to the whole blood staining identifying IL-6 receptor expression, this method did not involve the use of CD62L. For this reason, the memory CD4⁺ T-cell population will consist of a combination of the central memory and effector memory subpopulations. Furthermore, there is the potential for the naïve CD4⁺ T-cell subpopulation to contain CD45RA⁺CD62L⁻, terminally differentiated cells. These cells are only found in a small proportion of individuals, are found at extremely low numbers and have decreased proliferative capacity (457). These cells do however display potent cytotoxic activity following activation so this must be taken into account when interpreting the data from naïve CD4⁺ T-cells.

Basal phenotype of naïve and memory CD4⁺ T-cells was also examined in terms of co-stimulatory molecules, including CD27 and CD28. These have always been known as markers of antigen experience, however, the expression of these molecules is now realised to be more complex and may represent distinct populations of cells.

In keeping with previous studies our data confirmed CD27 is expressed by all naïve CD4⁺ T-cells whereas it is only found on around 80% of memory CD4⁺ T-cells (454). It has been described that memory CD4⁺ T-cells lacking CD27 display high antigen recall responses and produce higher levels of effector cytokines compared to resting CD27⁺ cells which require co-stimulation for TCR triggering (454). It is suggested that loss of CD27 expression represent a functionally differentiated population, which occur following chronic antigen stimulation (458).

In this Chapter, it was seen that expression of CD28 is maximal on both naïve and memory CD4⁺ T-cells. Cells which express CCR7, including naïve and central memory CD4⁺ T-cells, have been reported to have high levels of expression of CD28 (459). In contrast, effector memory CD4⁺ T-cells lacking CCR7 can be divided by their differential expression of CD28. Effector memory cells lacking CD28 expression correlate with those cells lacking CD27 expression and are associated with increased expression of cytolytic molecules and the predominant production of IFN- γ . It is now suggested that cells lacking CCR7 should not be considered to be a single population of cells and likely contains many subgroups based on both CD27 and CD28 expression. This population of memory CD28⁻CD4⁺ T-cells was not seen in cells isolated in the work outlined in this Chapter, however, this may be due to the process of isolation resulting in altered expression of this molecule.

3.4.3 Strengths of the in vitro model

In this study, it was important to optimise a method of isolation, which would provide a high yield of pure CD45RA⁺ naïve CD4⁺ T-cells. One of the strengths of this model is the method of isolation chosen which involves enrichment of CD4⁺ T-cells followed by depletion of CD45RO memory CD4⁺ T-cells resulting in a population of untouched naïve CD4⁺ T-cells.

Another strength of the in vitro model is the reductionist approach of the cell culture system. This approach aimed to establish a simplified in vitro model to limit the number of confounding factors in the system which may mask the effects of IL-6 pre-exposure. This meant that cells were cultured in serum free media TexMACS to avoid non-specific signalling in cells as a result of culture which have the potential to conceal the effects of IL-6 pre-exposure. Cells were also cultured in the absence or presence of IL-6 with equimolar concentrations of sIL-6R and without the refreshment of cytokines as this was revealed to have no effect on cell viability or surface marker expression. The addition of the sIL-6R allows both the classical and trans signalling mechanisms thereby encompassing both the anti- and pro- inflammatory properties of IL-6. The effect of sIL-6R in the system is further explored in *Chapter 4*.

The reductionist approach of this in vitro system allows the consequences of IL-6 pre-exposure to be directly probed with fewer confounding factors. Although the approach of refreshing cytokines could be considered to be more physiological and may have impacted the magnitude of differential phenotype following downstream TCR activation, this is an example of where the balance between reductionism and the approximation to the physiological state results in compromise hence resulting in unavoidable deviation from physiology.

As the work outline in thesis aims to investigate the effects of IL-6 pre-exposure on effector phenotype, it is important that IL-6 is no longer present at the time of TCR stimulation. To achieve this cells are thoroughly washed twice before stimulation. The stimulation of naïve and memory CD4⁺ T-cells with anti-CD3 and anti-CD28 and measurement of proliferation using CFSE allows for a more controlled level of stimulation which will determine a clear functional readout. Although stimulation with mature DCs is a more physiologically relevant stimulus as skewing cytokines will be provided by the DC this method resulted in high level of variability potentially due to biological variation (data not shown). The use of anti-CD3 and anti-CD28 ensures a quantifiable stimulus and permits the assessment of stimulation strength as a covariate influencing response of naïve CD4⁺ T-cells to IL-6 pre-exposure. The use of CFSE to track cell proliferation also allows more detailed analysis as it allows the tracking of the number of cell divisions and the number of cells in the starting population that underwent division.

3.4.4 Weaknesses of the in Vitro Model

Despite one of the strengths of the in vitro model being the isolation of large numbers of highly pure naïve CD4⁺ T-cells one of the weaknesses of this system is that this comes via the positive selection of memory CD4⁺ T-cells. The targeting of the CD45RO co-receptor may result in non-specific stimulation of the memory CD4⁺ T-cells and this has an unknown impact on downstream effector phenotype in terms of both gene expression and cell activation. Despite the binding of antibodies to co-receptors, it has been shown that isolation technique results in no significant differences in the gene expression profiles of cells or proliferative responses (460).

In addition to the positive selection of memory CD4⁺ T-cells, the purity of naïve CD4⁺ T-cells, typically over 80%, may also be considered a weakness. Decreased cell purity can result in inferior results due to the contamination by CD14 monocytes (461). These cells had low levels of monocyte contamination, avoided with the enrichment technique, to ensure more accurate gene expression analysis. Contaminating populations were largely naïve and memory CD4⁺ T-cells ensuring confidence that any resulting effects of IL-6 pre-exposure can be largely delineated to the CD4⁺ T-cell population. However, caution needs to be taken when interpreting data from naïve and memory CD4⁺ T-cells isolated in this way.

Furthermore, in the case of CD8⁺ T-cells when naïve and memory cells are cultured together it has been shown that antigen experienced T-cells may drive the differentiation of naïve cells to result in synchronising behaviours of the cells which were shown to affect the transcriptional, metabolic and functional processes of the naïve cells (462). For this reason, it was important to isolate a highly pure population of cells. Cell sorting was not feasible for this study due to timing and cost. Large volumes of blood would be needed for the isolation of naïve CD4⁺ T-cells which would take a long time to isolate via cell sorting in addition to the optimisation of a flow cytometry panel for their selection. Numerous cell isolations were required to obtain repeats in addition to analysis of different parameters following IL-6 pre-exposure, which meant this technique was too costly.

Another weakness of the in vitro model is the length of cell culture. The length of IL-6 pre-exposure was assessed after optimisation of culture media, culture with IL-6 and sIL-6R and the refreshment of cytokines. Due to this, these experiments all involved a culture period of 10 days prior to stimulation. This has been shown to result in lower levels of cell viability hence if length of culture was titrated prior to the optimisation of these parameters these experiments may have yielded different conclusions. Furthermore, many of the optimisation experiments carried out in this Chapter had limited numbers of repeats which may also have affected the

conclusions drawn, however in many of the cases when differences occurred as a result of culture with IL-6 these differences were clear.

Finally, the use of anti-CD3 and anti-CD28 antibodies for stimulation to mimic APC could be considered a weakness. The cytokine production by the APC is an important signal for T-cell effector function and this important component is missing from this reductionist in vitro model. Therefore, skewing of T-cells towards different T-helper cell subsets and hence effects on T-cell cytokine production may not be seen. This will be further investigated in *Chapter 4*.

3.4.5 Summary

To conclude, in this Chapter an optimised in vitro model of IL-6 pre-exposure has been developed, which can be seen in *Figure 23*. This model involves the enrichment of CD4⁺ T-cells from blood followed by isolation of naïve CD4⁺ T-cells using CD45RO MicroBeads. Cells are CFSE labelled prior to culture with varying concentrations of IL-6 and equimolar concentrations of sIL-6R for 3 days. After 3 days, cells are thoroughly washed to remove IL-6 from the culture media and cells stimulated for a further 6 days with both a high stimulus and a low stimulus using anti-CD3 and anti-CD28 antibodies. This in vitro system will permit a detailed investigation into the consequences of prolonged pre-exposure to IL-6. This will be assessed by its ability to influence effector phenotype following TCR mediated activation in terms of proliferation, activation and cytokine production and resulting dynamic transcriptional effects, *Chapter 4* and *Chapter 5*, respectively.

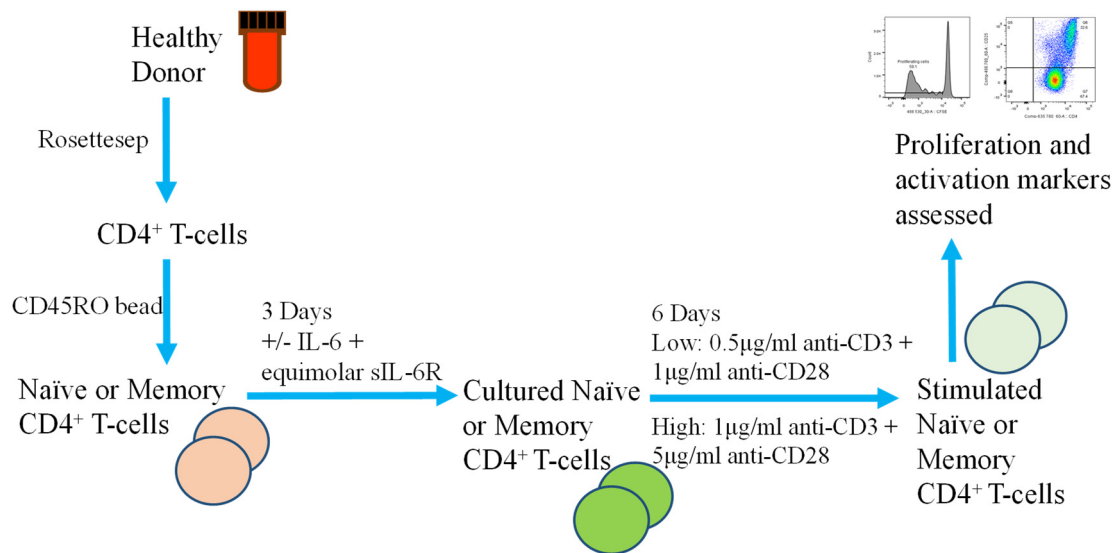


Figure 23. Optimised in vitro model of IL-6 pre-exposure of naïve CD4⁺ T-cells. CD4⁺ T-cells are isolated by RosetteSep enrichment. Naïve CD4⁺ T-cells are further isolated by depletion of memory CD4⁺ T-cells using CD45RO microbeads. Naïve or memory CD4⁺ T-cells are labelled with a final concentration of 0.5µM CFSE and cultured for 3 days in TexMACS alone, TexMACS with 0.1ng/ml IL-6, TexMACS with 0.5ng/ml IL-6 or TexMACS with 50ng/ml IL-6 all with equimolar sIL-6R. After 3 days cells are washed to remove IL-6 and sIL-6R and cultured with 1µg/ml anti-CD3 and 5µg/ml anti-CD28 (high) or 0.5µg/ml anti-CD3 and 1µg/ml anti-CD28 (low) for a further 6 days. Proliferation and activation markers are assessed by flow cytometry.

Chapter 4. Effects of IL-6 pre-exposure on naïve and memory CD4⁺ T-cell effector function

4.1 Introduction

It has been long understood that antigen-independent, cytokine mediated activation of T-cells can occur in vivo (463). A combination of cytokines, including IL-6, are able to induce the proliferation and effector function of CD4⁺ T-cells in the absence of TCR stimulation (463). Furthermore, evidence for the “sensitising” or “priming” of a number of different cells by a range of pro-inflammatory cytokines has been more recently described. Mesenchymal stem cells (MSCs) can be primed towards an anti-inflammatory phenotype by the presence of IL-1 prior to stimulation (464). In contrast, neutrophils primed with GM-CSF, IFN- γ and TNF- α have increased inflammatory cytokine production (465). Cells of the adaptive immune system have also been shown to have this “pre-priming” potential as CD4⁺ T-cells have been shown to respond aberrantly to chronic TNF- α exposure by the attenuation of TCR signalling and the suppression of proliferation and cytokine production (466). These results suggest that immune cells have potential memory of the inflammatory environment, which can programme them towards altered effector phenotypes following activation.

It has been clearly shown that IL-6 is present at high concentrations in RA patients at the time of disease presentation and it has been further shown that IL-6 can be found at high levels long before the clinical onset of arthritis (467-469). There has been no specific disease associated autoantigen which has been found to account for immune dysregulation in RA suggesting it is more of a pro-inflammatory than autoimmune disease. Given that naïve CD4⁺ T-cells are strikingly sensitive to IL-6 at the level of STAT-3 signalling, shown to be uniquely up regulated in early RA, here it is hypothesised that this is an important mechanism of antigen non-specific adaptive immune dysregulation early in the disease setting (427).

In *Chapter 3* an in vitro system was developed in order to recapitulate the chronic exposure of naïve and memory CD4⁺ T-cells to IL-6. In this in vitro model naïve and memory CD4⁺ T-cells are isolated before pre-exposure to varying concentrations of IL-6 for 3 days. IL-6 is then washed out of the system prior to TCR stimulation. High levels of IL-6 can be found in RA and it is likely that in vivo IL-6 is present at the time of stimulation, however; this reductionist approach allows the investigation of the extent to which IL-6 pre-exposure is mechanistically important to CD4⁺ T-cell effector function.

This Chapter first assessed the ability of IL-6 and other pro-inflammatory and STAT-3 signalling cytokines to induce the three key STAT-3 regulated genes found to be dysregulated

in early RA, to reveal how specific this effect is to IL-6. Next, the consequences of IL-6 pre-exposure on the effector functions of naïve and memory CD4⁺ T-cells following TCR mediated activation were investigated.

IL-6 signalling has profound effects on T-cell responses, specifically causing T-cell activation, inhibition of apoptosis, increased cell expansion, survival and infiltration (470-472). Due to this, the effect of IL-6 pre-exposure on naïve and memory CD4⁺ T-cell survival, activation and proliferation was measured. Several phenotypic surface markers were assessed following IL-6 pre-exposure including CD25 and CD40L, which are upregulated early during the activation of CD4⁺ T-cells. In addition to these markers CXCR5 and latency associated peptide (LAP) expression were also analysed. LAP is the membrane bound form of TGF- β 1, included as a marker of production of TGF- β , a difficult cytokine to measure via ELISA of supernatants or via intracellular cytokine staining. Based on previous work this marker has also been found to be strongly upregulated in response to strong stimulation via the TCR (Amy Anderson, Newcastle University, unpublished data) so this marker was included to assess if there was any influence of IL-6 pre-exposure. CXCR5 is the key chemokine receptor expressed on Tfh cells. IL-6 has been heavily implicated in the development of Tfh cells by the induction of IL-21. IL-6 also upregulates BCL-6 which has been shown to induce the master transcription factor of Tfh cells, BCL-6 (164). Expression levels of this gene have been shown to correlate with Tfh cell related genes such as ICOS and CXCR5. Hence, pre-exposure of CD4⁺ T-cells to IL-6 may result in increased expression of Tfh cell markers. Furthermore, circulating Tfh cells have been found to be increased in early RA suggesting IL-6 pre-exposure may have a pathophysiological role in the development of these cells (473).

The presence of IL-6 during TCR stimulation is linked to increased Th2 and decreased Th1 differentiation (363, 474). IL-6 may also influence but is not the only cytokine required for commitment to the Th17 and Tfh cell lineages (364, 386) whilst trans signalling abrogates the induction of Tregs (371). Due to this complex role of IL-6 in T-helper cell differentiation the expression of master transcription factors for Th1, Th2, Th17 and Tregs was analysed as well as the effects of IL-6 pre-exposure on cytokine production and subsequent T-helper cell differentiation.

All of these factors were assessed in order to determine the consequences of IL-6 pre-exposure on downstream CD4⁺ T-cell effector phenotype.

4.2 Aims and Objectives

The aim of this chapter was, using the in vitro system developed in *Chapter 3*, to investigate the consequences of prolonged IL-6 pre-exposure upon naïve and memory CD4⁺ T-cell effector function following TCR mediated activation. In order to achieve this the proliferation and activation of cells was assessed post TCR stimulation. Cytokine production and transcription factor expression was assessed with IL-6 pre-exposure in addition to examining the influence of this pre-exposure on T-helper cell differentiation.

The specific objectives of this chapter were:

- Investigation into the specificity of IL-6 in the induction of the three key STAT-3 regulated genes dysregulated in early RA
- Determination of the consequences of sustained IL-6 pre-exposure on naïve and memory CD4⁺ T-cell effector function following TCR mediated activation, in terms of proliferation, activation and cytokine production.
- Determination of whether the effect of IL-6 pre-exposure on naïve and memory CD4⁺ T-cell effector phenotype is related to the IL-6 pre-exposure dose or duration.
- Exploration of the effect of chronic IL-6 pre-exposure of naïve and memory CD4⁺ T-cells on subsequent transcription factor expression and T-helper cell differentiation.

4.3 Results

4.3.1 IL-6 induces STAT-3 target genes

The previously described 12-gene transcriptional signature, which has the potential to discriminate early RA patients, contains a number of STAT-3 target genes, the most differentially expressed of these being BCL3, PIM1 and SOCS3 (431). Serum levels of IL-6 were shown to correlate with expression of these genes as well as with levels of constitutively activated STAT-3 (427). I sought to assess whether, in an in vitro setting, IL-6 could induce the same combination of STAT-3 regulated genes and, if so, the extent to which this effect was specific to IL-6 or common to other pro-inflammatory cytokines. Total CD4⁺ T-cells were isolated from healthy donors by positive selection and incubated for 6 hours with a range of pro-inflammatory cytokines. RNA was isolated at each time point, reverse transcribed and the expression of BCL3, SOCS3 and PIM1 was examined by RT-PCR.

Culture of CD4⁺ T-cells with a supra-physiological concentration of 50ng/ml IL-6 caused induction of SOCS3 and PIM1 with induction around 10 fold and 2 fold, whereas there appears to be no induction of BCL-3 *Figure 24*. Culture of CD4⁺ T-cells with pro-inflammatory

cytokine TNF- α resulted in no induction of any of the STAT-3 mediated genes, *Figure 24A*, whereas IFN- α strongly induced PIM1 but had no effect on BCL-3, *Figure 24C*. The stimulation of CD4⁺ T-cells with other STAT-3 signalling pro-inflammatory cytokines (IL-21, IL-23, IL-27) resulted in a pattern of STAT-3 target gene induction more reminiscent of that of IL-6, with good induction of SOCS3 by IL-21 and IL-27 however only in the case of IL-21 was this consistent for all 3 genes examined, *Figure 24*. This suggests IL-21, in addition to IL-6, has the ability to generate the STAT-3 gene signature described in early RA.

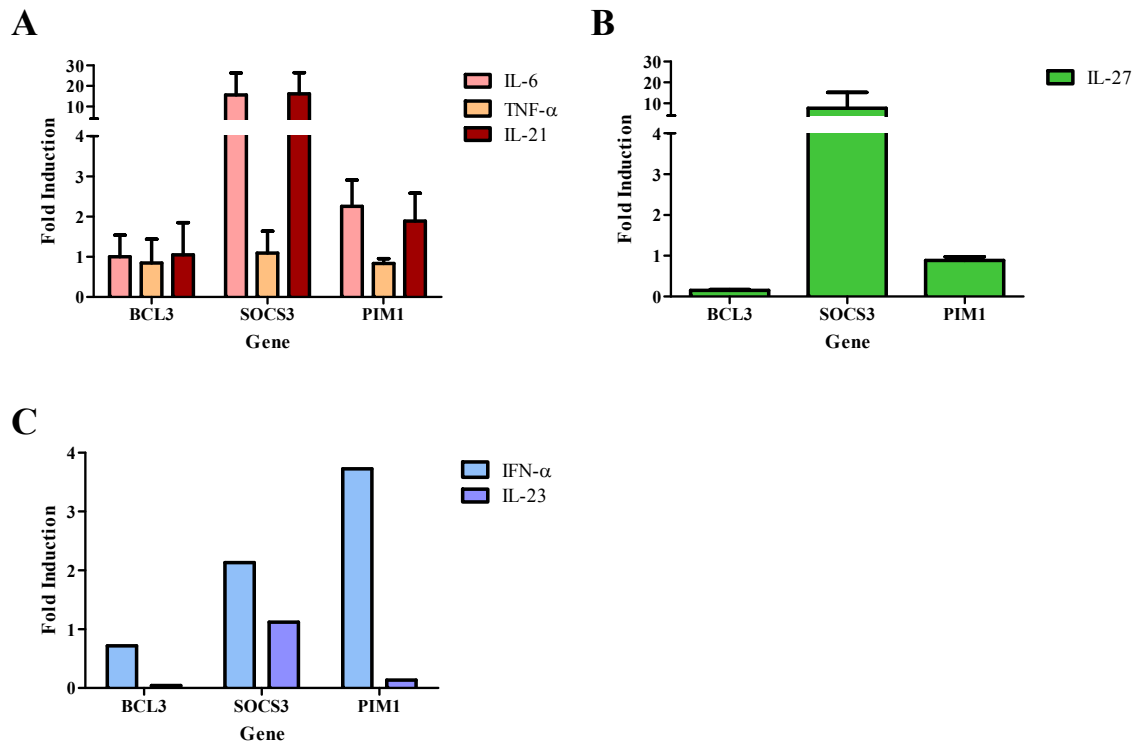


Figure 24. Fold induction of STAT-3 regulated genes in CD4⁺ T-cells stimulated with pro-inflammatory cytokines. Freshly isolated CD4⁺ T-cells were cultured for 6 hours in TexMACS alone, or with 50ng/ml final concentrations of a range of cytokines including; IL-6 and equimolar sIL-6R, TNF- α , IL-21 (A), IL-27 (B), IFN- α and IL-23 (C). Following stimulation cells were harvested, RNA was isolated and expression of STAT-3 regulated genes BCL3, PIM1 and SOCS3 were assessed using TaqMan real time PCR. Δ CT values of each sample were determined by normalising to the housekeeping gene 18S. Fold induction was calculated by dividing $2^{-\Delta$ CT of cytokine stimulated cells by $2^{-\Delta$ CT of untreated cells, this is equivalent to $2^{\Delta\Delta$ CT}. Values from 3 (A), 2 (B) or 1 (C) biological replicates were plotted on a graph with bars representing mean values and error bars showing SEM

To summarise, IL-6 is a clear inducer of SOCS3 and PIM1 however failed to induce BCL-3 in this experiment. Other pro-inflammatory cytokines or STAT-3 cytokines were able to induce either SOCS3 or PIM1 with IL-21 reflecting similar induction to IL-6. Further investigation is needed into the effects of stimulation with IL-6 and IL-21.

4.3.2 Viability of naïve and memory CD4⁺ T-cells following IL-6 pre-exposure

The next aim of this Chapter was determine if pre-exposure to IL-6 could alter effector phenotype with the first aim to determine whether IL-6 pre-exposure could increase cell survival. Naïve and memory CD4⁺ T-cells were cultured with varying concentrations of IL-6 and equimolar concentrations of sIL-6R and stimulated as per the in vitro model, *Chapter 3.4.5*. Viability was assessed by staining with DAPI for flow cytometry. The gating strategy for this analysis can be seen in *Figure 5, Appendix*.

There was no clear difference in viability of naïve or memory CD4⁺ T-cells pre-exposed to IL-6 following 6 days of TCR stimulation, with 80% viability for all conditions, *Figure 25*. This high viability suggests that viability is not a confounding factor in the subsequent functional outputs measured. Although IL-6 has been indicated to increase CD4⁺ T-cell viability, in this case IL-6 has no long lasting effects on naïve or memory CD4⁺ T-cell viability (470).

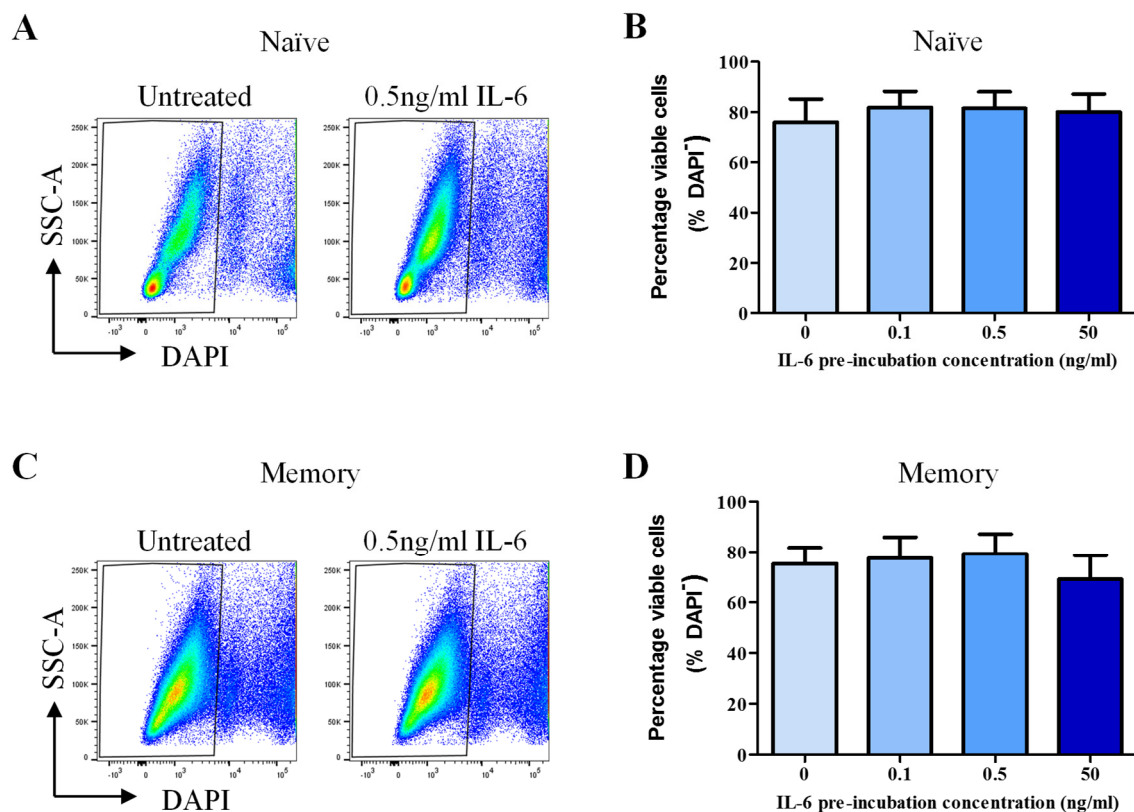


Figure 25. Viability of naïve and memory CD4⁺ T-cells following pre-exposure to IL-6 and subsequent TCR stimulation. Freshly isolated naïve and memory CD4⁺ T-cells were cultured for 3 days in TexMACS alone, with 0.1ng/ml IL-6, 0.5ng/ml IL-6 or 50ng/ml IL-6 all with equimolar sIL-6R. After 3 days naïve (**A and B**) and memory (**C and D**) cells were washed and stimulated with 0.5µg/ml anti-CD3 and 1µg/ml anti-CD28 for a further 6 days. Following stimulation cells were stained with DAPI for flow cytometry and assessment of viability. Viable cells were gated as DAPI⁻. Representative dot plots are shown for naïve (**A**) and memory CD4⁺ T-cells (**C**). Values from naïve (**B**) and memory CD4⁺ T-cells (**D**) from 5 biological replicates were plotted on a graph with bars showing median and error bars representing SEM.

4.3.3 IL-6 pre-exposure causes increased proliferative capacity following TCR stimulation

Although there was no effect of IL-6 pre-exposure on cell viability the next aim was to assess whether pre-exposure to IL-6 could alter effector phenotype in terms of proliferative capacity. Naïve and memory CD4⁺ T-cells were isolated from LRS cones and cultured with varying concentrations of IL-6 and equimolar sIL-6R. Cells were washed and subsequently stimulated for 3 or 6 days with plate-bound anti-CD3 and soluble anti-CD28 at 1µg and 5µg respectively (termed “high stimulus”) and proliferation was measured by CFSE and the FlowJo proliferation tool. The gating strategy for all proliferation analyses can be seen in *Figure 9, Chapter 2.9.5*.

High stimulus with anti-CD3 and anti-CD28 induces robust levels of proliferation at 6 days, with higher levels of proliferation seen in memory CD4⁺ T-cells compared to naïve CD4⁺ T-cells with the percentage of cells dividing reaching levels between 10-25% or 10-17% respectively, *Figure 26A and B*. A clear increase in proliferation can be seen as a consequence of pre-exposure to increasing concentrations of IL-6, significant by non-parametric analysis of variance, $p < 0.05$. However, Dunn’s posthoc analysis of IL-6 pre-exposed naïve and memory CD4⁺ T-cells compared to non-exposed cells revealed only pre-exposure to supra-physiological concentrations of IL-6 (50ng/ml) and equimolar sIL-6R causes significantly increased levels of proliferation compared to the non-pre-exposed cells, *Figure 26*.

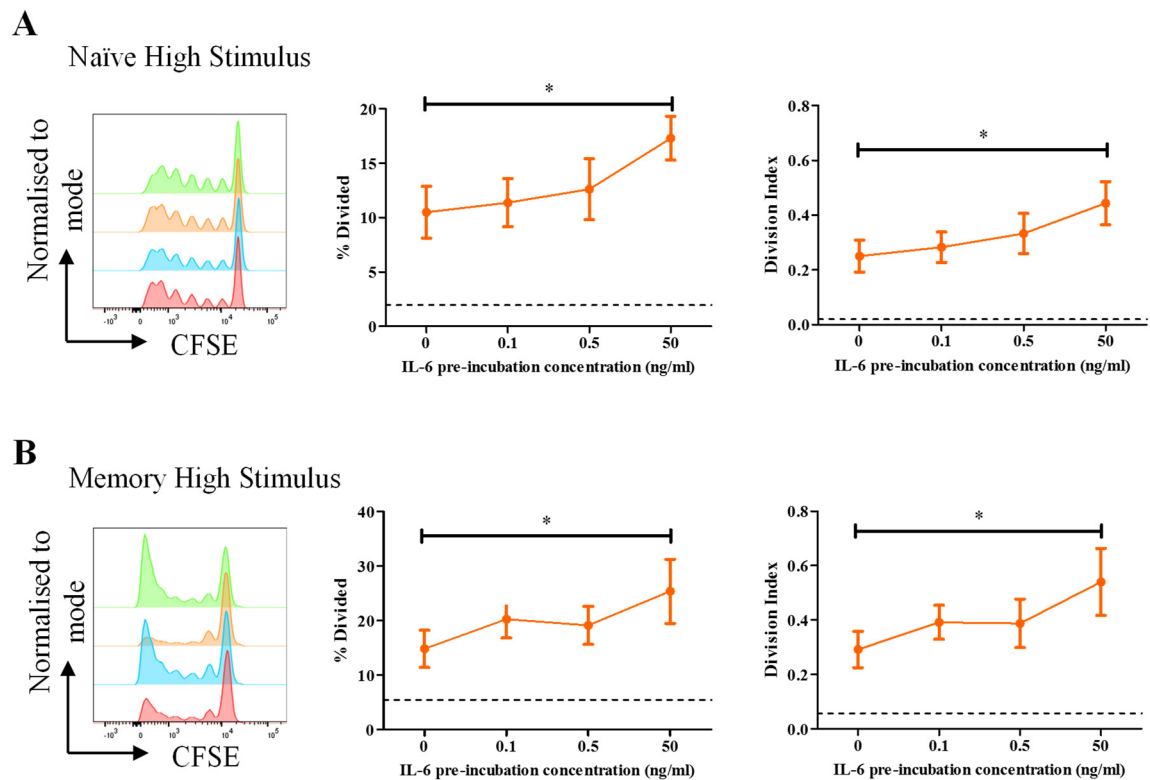


Figure 26. Proliferation of naïve and memory CD4⁺ T-cells following pre-exposure to IL-6 and 6-day high TCR stimulation. Freshly isolated CFSE labelled naïve (A) and memory (B) CD4⁺ T-cells were cultured for 3 days in TexMACS alone, with 0.1ng/ml, 0.5ng/ml or 50ng/ml IL-6 all with equimolar sIL-6R. Cells were then washed and stimulated with; 1µg/ml anti-CD3 and 5µg/ml anti-CD28 (high stimulus) for a further 6 days. Cells were acquired on the flow cytometer. Cells were gated as singlets, lymphocytes and proliferating cells were identified as DAPI⁻. Proliferation was calculated using FlowJo, shown in *Figure 9, Chapter 2.9.5*, with percentage divided indicating the precursor frequency of dividing cells and division index showing the average number of divisions, with representative plots of CFSE histograms from one donor shown in the left hand panel. Values from 5 biological replicates were plotted as graphs with points showing median and error bars representing SEM. Non-parametric analysis of variance (Friedman) demonstrated a significant effect in naïve and memory CD4⁺ T-cells irrespective of proliferation parameter (*p<0.05 in each case). Comparisons of 50ng/ml, 0.5ng/ml and 0.1ng/ml IL-6 pre-exposed cells with non-pre-exposed cells were undertaken using Dunn's post hoc pairwise analysis; *p<0.05.

As it is possible that the higher concentration stimulus may be inducing maximal levels of proliferation and thus masking the consequences of IL-6 pre-exposure the effect of IL-6 pre-exposure on naïve and memory CD4⁺ T-cells was next explored following stimulation with 0.5µg anti-CD3 and 1µg anti-CD28 ("low stimulus"). The low stimulus induced lower levels of proliferation compared to the high stimulus with the percentage of cells dividing ranging from 5-20%, *Figure 27*. Once again, there was a clear increase in proliferation of naïve and memory CD4⁺ T-cells as a consequence of pre-exposure to increasing concentrations of IL-6, significant by non-parametric ANOVA, p<0.05. As 0.5ng/ml IL-6 and equimolar sIL-6R is a

concentration which mirrors the levels found in the upper limits of that seen in early RA patients, and as such was deemed more physiologically relevant, it was explored a priori whether this concentration could significantly increase proliferation compared to untreated cells. In a single Dunn's posthoc test there was significant increase in proliferation of both naïve and memory CD4⁺ T-cells from the untreated condition, following pre-exposure to 0.5ng/ml IL-6 and equimolar sIL-6R with the low stimulus, *Figure 27*. However, Dunn's posthoc analysis of all IL-6 pre-exposed naïve and memory CD4⁺ T-cells compared to non-exposed cells revealed only pre-exposure to supra-physiological concentrations of IL-6 (50ng/ml) and equimolar sIL-6R causes significantly increased levels of proliferation compared to the non-pre-exposed cells, *Figure 27*. From these analyses, it can be seen that the proliferative capacity of CD4⁺ T-cells increases significantly 6 days post anti-CD3 and anti-CD28 stimulation in a manner that is dose-dependent with respect to IL-6 pre-exposure.

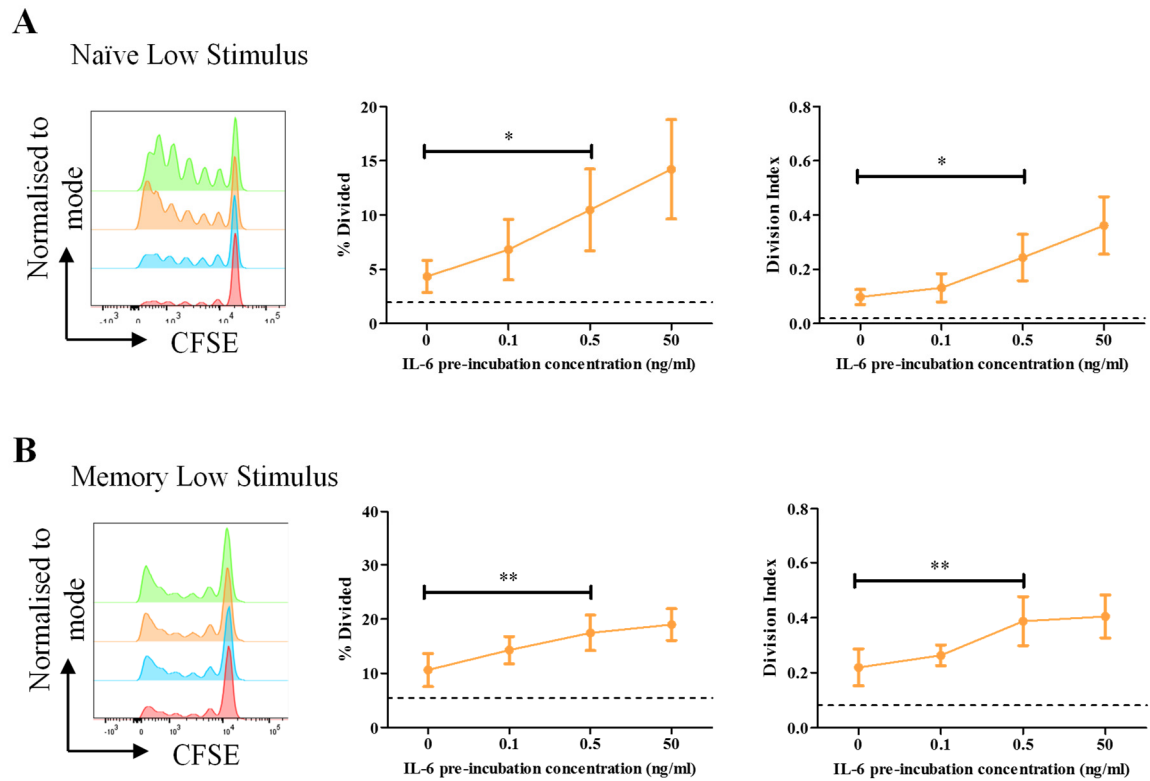


Figure 27. Proliferation of naïve and memory CD4⁺ T-cells following pre-exposure to IL-6 and 6-day low TCR stimulation. Freshly isolated CFSE labelled naïve (A) and memory (B) CD4⁺ T-cells were cultured for 3 days in TexMACS alone, with 0.1ng/ml, 0.5ng/ml or 50ng/ml IL-6 all with equimolar sIL-6R. Cells were then washed and stimulated with; 0.5µg/ml anti-CD3 and 1µg/ml anti-CD28 (low stimulus) for a further 6 days. Cells were acquired on the flow cytometer. Cells were gated as singlets, lymphocytes and proliferating cells were identified as DAPI⁻. Proliferation was calculated using FlowJo, shown in *Figure 9, Chapter 2.9.5*, with percentage divided indicating the precursor frequency of dividing cells and division index showing the average number of divisions, with representative plots from one donor shown in the left hand panel. Values from 5 biological replicates were plotted with points representing the median and error bars representing SEM. Non-parametric analysis of variance (Friedman) demonstrated a significant effect in naïve and memory CD4⁺ T-cells irrespective of proliferation parameter (*p<0.05 in each case). Comparisons of 0.5ng/ml IL-6 pre-exposed cells with non-pre-exposed cells were undertaken in a single Dunn's posthoc pairwise analysis, p<0.05, ** p<0.005.

As an increase in proliferation was observed following pre-exposure to IL-6 and equimolar sIL-6, the extent to which the observations described on proliferation were dependent on the presence of sIL-6R (and hence, potentially, the trans signalling mechanism) was next considered. Naïve CD4⁺ T-cells were isolated and cultured as previously described with varying concentrations of IL-6 with or without equimolar sIL-6R prior to TCR stimulation. In this experiment the low stimulus was used as this better represents the differences seen in proliferation with IL-6 pre-exposure. Again, there was increased proliferation with increasing concentrations of IL-6 pre-exposure seen with IL-6 and sIL-6R and this was also apparent in

the absence of sIL-6R, significant by non-parametric ANOVA with $p < 0.05$, *Figure 28*. . In a single Dunn's posthoc test there was significant increase in proliferation of naïve $CD4^+$ T-cells from the untreated condition only following pre-exposure to 0.5ng/ml IL-6 and equimolar sIL-6R but this was not significant in the absence of sIL-6R, *Figure 28*. However, Dunn's posthoc analysis of all IL-6 pre-exposed naïve $CD4^+$ T-cells compared to non-exposed cells revealed pre-exposure to supra-physiological concentrations of IL-6 (50ng/ml) without the presence of equimolar sIL-6R causes significantly increased levels of proliferation compared to the non-pre-exposed cells. It appears that without the addition of sIL-6R there is an increase in the number of cells dividing and the number of divisions compared to cells cultured with sIL-6R can only be seen at the highest concentration of IL-6, 50ng/ml compared to the untreated condition. However, the presence of the sIL-6R significantly increased levels of proliferation can be seen with 0.5ng/ml IL-6 pre-exposure, *Figure 28A-B*.

Finally it was confirmed that proliferating cells were $CD3^+CD4^+$ T-cells, with less than 1% of cells in culture being monocytes, B-cells or NK cells, and no such contaminating cells were present in the diluted CFSE populations post-stimulation (*Figure 14, Appendix*).

To summarise, the observed dose-dependent increases in proliferation relate specifically to of naïve and memory $CD4^+$ T-cells. In addition, although increased proliferation appears to be seen with pre-exposure to IL-6 alone only when cultured with sIL-6R is a significant increase in proliferation seen, indicating a role for trans signalling.

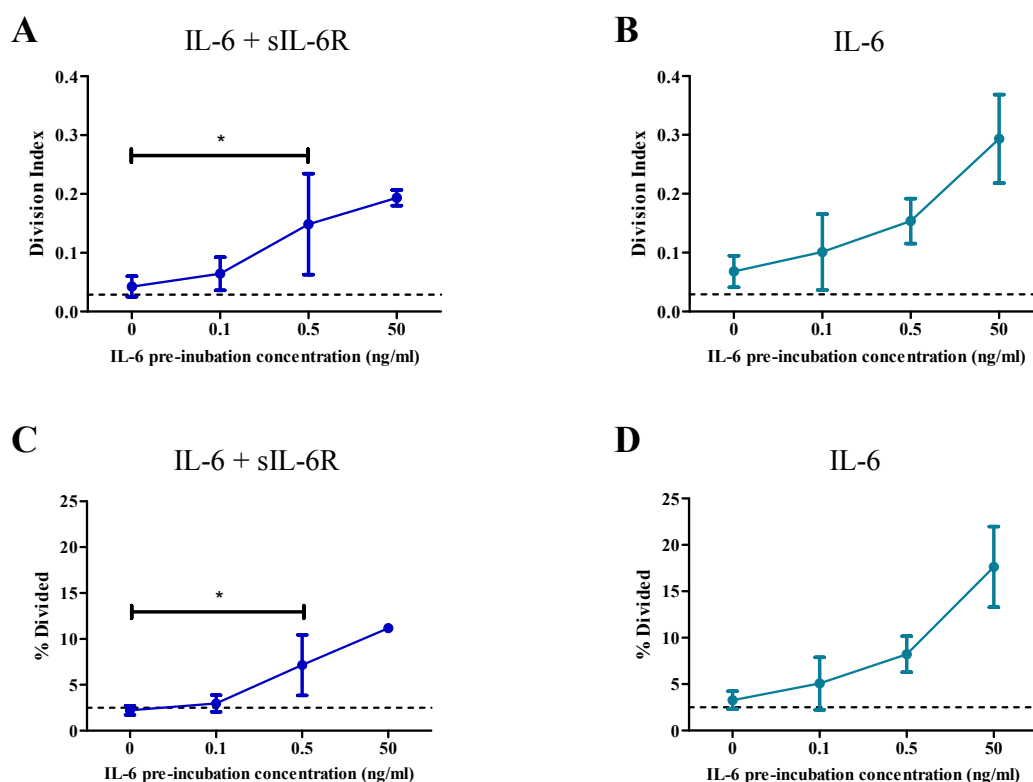


Figure 28. Proliferation of naïve CD4⁺ T-cells following pre-exposure to IL-6 with or without sIL-6R and subsequent TCR stimulation. Freshly isolated CFSE labelled naïve CD4⁺ T-cells were cultured for 3 days in TexMACS alone, with 0.1ng/ml, 0.5ng/ml or 50ng/ml IL-6 all with (A and C) or without (B and D) equimolar sIL-6R. After 3 days cells were washed and stimulated with 0.5µg/ml anti-CD3 and 1µg/ml anti-CD28 for a further 6 days. Following stimulation cells were acquired on the flow cytometer. Cells were gated as singlets, lymphocytes and proliferating cells were identified DAPI⁻. Proliferation was calculated using FlowJo, shown in *Figure 9, Chapter 2.9.5*, with division index showing the average number of divisions (A and B). and percentage divided indicating the precursor frequency of dividing cells (C and D), Values from 3 biological replicates were plotted with points showing median and error bars representing SEM. Non-parametric analysis of variance (Friedman) demonstrated a significant effect in both the presence or absence of sIL-6R (*p<0.05 in each case). Comparisons of 0.5ng/ml IL-6 pre-exposed cells with non-pre-exposed cells were undertaken in a single Dunn's posthoc pairwise analysis, p<0.05.

4.3.4 Increased proliferative capacity is lost with reduced IL-6 pre-exposure

As pre-exposure of naïve and memory CD4⁺ T-cells resulted in increased proliferative capacity it was next investigated to what extent the effect of IL-6 pre-exposure on increased proliferative capacity was dependent upon the duration of IL-6 pre-exposure. Naïve and memory CD4⁺ T-cells, isolated from LRS cones, were cultured with varying concentrations of IL-6 and equimolar sIL-6R for 1 hour, 16 hours, 48 hours and 72 hours. At each of these points, cells were washed and stimulated as per the in vitro model, *Chapter 3.4.5*, and proliferation assessed after 6 days.

The proliferative response after the usual 72 hours pre-exposure to IL-6 followed by 6 day stimulation shows dose-dependent increase in proliferation with increasing concentrations of IL-6 pre-exposure. This trend is still apparent after 48 hours pre-exposure to IL-6 followed by stimulation, however, the trend is completely abrogated with only 16 hours or 1 hour of IL-6 pre-exposure, *Figure 29*. This pattern occurred in both naïve CD4⁺ T-cells and memory CD4⁺ T-cells, *Figure 15-16, Appendix*. A two-way ANOVA confirmed the interaction between pre-exposure time and IL-6 concentration as significantly different, $p < 0.05$, *Figure 29*. This highlights that the effect of IL-6 pre-exposure on proliferative capacity depends on its duration and becomes apparent only following more pro-longed pre-exposures.

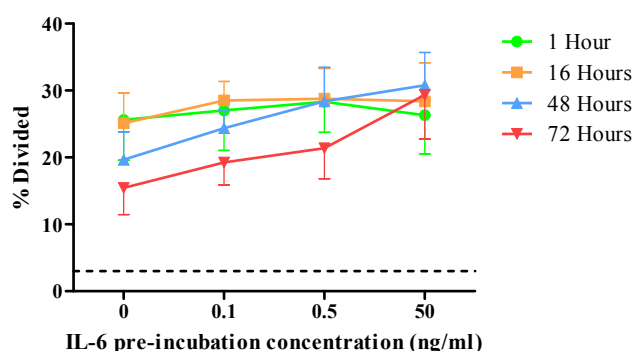


Figure 29. Proliferation of naïve CD4⁺ T-cells following shortened pre-exposure to IL-6 and subsequent TCR stimulation. Freshly isolated CFSE labelled naïve CD4⁺ T-cells were cultured for 1 hour, 16 hours, 48 hours, or 72 hours in TexMACS alone, with 0.1ng/ml IL-6, 0.5ng/ml IL-6 or 50ng/ml IL-6, all with equimolar sIL-6R. After exposure to IL-6 naïve CD4⁺ T-cells were washed and stimulated with 0.5µg/ml anti-CD3 and 1µg/ml anti-CD28 for a further 6 days. Following stimulation cells were acquired on the flow cytometer. Cells were gated as singlets, lymphocytes and then proliferating cells were DAPI⁺. Proliferation was calculated using FlowJo, shown in *Figure 9, Chapter 2.9.5*, with percentage divided indicating the precursor frequency of dividing cells. Values from 3 biological replicates were plotted with points showing median and error bars showing SEM, dotted line represents maximal proliferation of unstimulated cells. Significance was determined by two-way ANOVA, $*p < 0.05$.

Overall, these experiments confirm the dependency of the effects seen on effector phenotype on the “chronicity” of IL-6 pre-exposure in vitro.

4.3.5 IL-6 pre-exposure causes increased activation status following TCR stimulation

After observing an increase in naïve and memory CD4⁺ T-cell proliferative capacity following pre-exposure to IL-6 the next parameter to be assessed was the effect of IL-6 pre-exposure on the expression of IL-6 signalling components and cell surface activation markers. To do this, naïve and memory CD4⁺ T-cells, isolated from LRS cones, were cultured with varying concentrations of IL-6 and equimolar sIL-6R prior to TCR stimulation, as previously described. Cell surface markers were assessed on day 6 post-TCR stimulation by staining with a panel of

antibodies for phenotype or activation for flow cytometry. For phenotype analyses gates were set employing the approach of setting gates on the starting population of CD4⁺ T-cells while for activation analyses gates were set based on unstained controls and in the case of CD40L with the use of FMO control, *Figures 3-4, Appendix*.

The expression of phenotypic markers and IL-6 signalling components were assessed on naïve CD4⁺ T-cells following culture for 3 days with varying concentrations of IL-6 and equimolar sIL-6R and subsequent 6 day stimulation with 1µg/ml anti-CD3 and 10µg/ml anti-CD28. This level is different to the dose optimised in vitro model, *Chapter 3.4.5* as it was carried out prior to determining optimal stimulation dose. It was found that post-TCR stimulation expression of CD25 doubled from baseline (*Figure 14*), whereas CD28 was dramatically decreased from baseline with no change in CD27 expression, *Figure 30*. In addition expression of IL-6R was reduced from baseline expression (*Figure 14*) whereas gp130 levels was increased from basal expression levels, *Figure 30*. Levels of expression do not appear to differ with differing concentrations of IL-6 showing the effect of IL-6 on the levels of these markers is lost following stimulation.

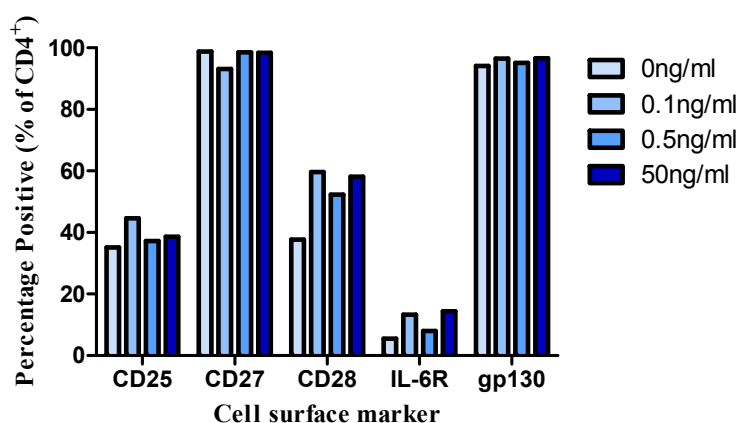


Figure 30. Expression of phenotypic markers and IL-6 signalling receptors on naïve CD4⁺ T-cells following pre-exposure to IL-6 and subsequent TCR stimulation. Freshly isolated naïve CD4⁺ T-cells were cultured for 3 days in TexMACS alone, with 0.1ng/ml IL-6, 0.5ng/ml IL-6 or 50ng/ml IL-6 all with equimolar sIL-6R. After 3 days cells were washed and stimulated with 1µg/ml anti-CD3 and 10µg/ml anti-CD28 for a further 6 days. Following stimulation cells were stained for cell surface markers for phenotype analysis. Cells were gated as singlets, lymphocytes and then CD4⁺ T-cells were identified as CD3⁺CD4⁺. N=1.

The expression of activation markers were assessed on naïve CD4⁺ T-cells following culture as per the in vitro model, *Chapter 3.4.5*. Representative histograms for each marker can be seen in *Figure 31*, which show the intensity of expression of each molecule and the number of cells with this intensity. This shows that there is no difference in expression of LAP or CXCR5 but there is a clear increase in the expression of CD40L. In addition, for CD25 it appears that with

pre-exposure to IL-6 a greater number of cells express CD25 and those that express CD25 also express a higher level of CD25. Data from dot plots showing the percentage of cells positive for each marker was used to generate bar charts showing the percentage of cells positive for that marker, representative dot plots can be seen in *Figure 17, Appendix*.

The percentages of cells positive for each marker was determined by dot plots, with representative plots in *Figure 17, Appendix*. Data from biological replicates was plotted as graphs for each marker. These show there was a significant effect with increasing concentrations of IL-6 pre-exposure for CD25 in naïve CD4⁺ T-cells and CD40L in both naïve and memory CD4⁺ T-cells, significant by non-parametric ANOVA with $p < 0.05$, *Figure 31*. In a single Dunn's posthoc test there was significant increase in the expression of CD25 and CD40L of naïve CD4⁺ T-cells from the untreated condition following pre-exposure to 0.5ng/ml IL-6 and equimolar sIL-6R. However, Dunn's posthoc analysis of all concentrations of IL-6 pre-exposure of naïve CD4⁺ T-cells compared to non-exposed cells revealed that for CD40L only pre-exposure to supra-physiological concentrations of IL-6 (50ng/ml) caused significantly increased expression of this molecule. For CD25 both 50ng/ml and 0.5ng/ml IL-6 pre-exposure resulted in significantly increased expression of this molecule. For memory CD4⁺ T-cells Dunn's posthoc analysis of all concentrations of IL-6 pre-exposure compared to non-exposed cells revealed that for CD40L pre-exposure to supra-physiological concentrations of IL-6 (50ng/ml) caused significantly increased expression of this molecule.

In summary, increased expression of activation markers CD25 and CD40L can be seen with increasing concentrations of IL-6 pre-exposure, 6 days post TCR stimulation amongst naïve CD4⁺ T-cells, and these effects are not observed amongst memory CD4⁺ T-cells.

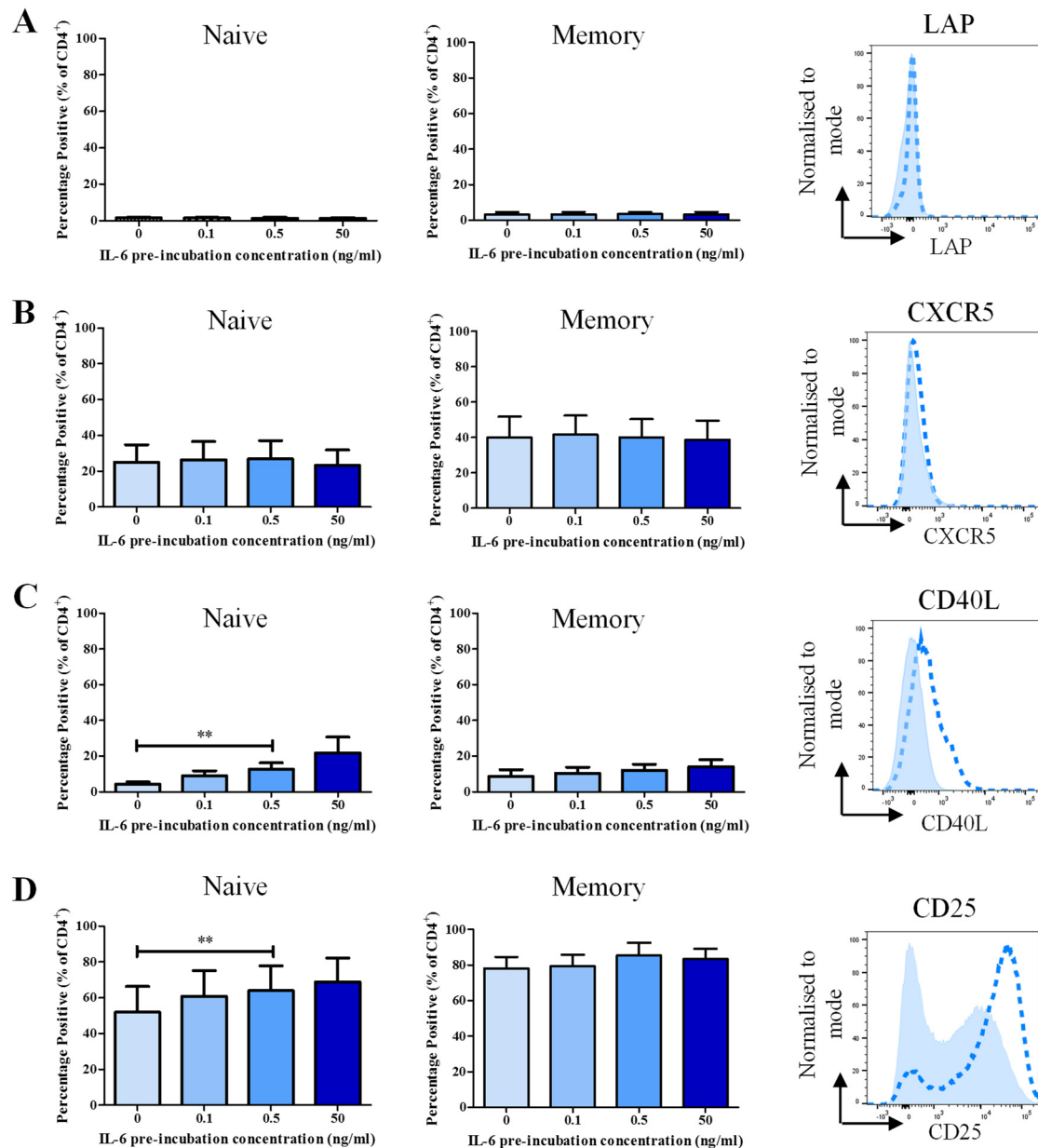


Figure 31. Expression of activation markers by naïve and memory CD4⁺ T-cells following pre-exposure to IL-6 and subsequent 6 day TCR stimulation. Freshly isolated naïve and memory CD4⁺ T-cells were cultured for 3 days in TexMACS alone, with 0.1ng/ml, 0.5ng/ml or 50ng/ml IL-6, all with equimolar sIL-6R. After 3 days cells were washed and stimulated with 0.5µg/ml anti-CD3 and 1µg/ml anti-CD28 for a further 6 days before staining for activation markers for flow cytometry. Cells were gated as singlets, lymphocytes and CD4⁺ T-cells were identified as CD3⁺CD4⁺. Expression of LAP (A), CXCR5 (B), CD40L (C) and CD25 (D) were measured for 3 (A), 5 (B), or 6 (C and D) biological replicates as percentage positive using dot plots and values plotted with bar representing the median and error bars representing SEM, the left panel shows naïve CD4⁺ T-cells and the middle panel shows memory CD4⁺ T-cells. The right panel shows representative histograms for untreated cells (pale blue shaded) and cells pre-exposed to 0.5ng/ml IL-6 (dark blue dashed line), showing intensities of expression of each molecule. Non-parametric analysis of variance (Friedman) demonstrated a significant effect for CD40L and CD25 in naïve CD4⁺ T-cells and for CD40L in memory CD4⁺ T-cells (**p<0.05 in each case). Comparisons of 0.5ng/ml IL-6 pre-exposed cells with non-pre-exposed cells were undertaken in a single Dunn's posthoc pairwise analysis, * p<0.05, ** p<0.005.

4.3.6 Cytokine production following IL-6 pre-exposure

Given that IL-6 pre-exposure resulted in clear functional and phenotypic consequences in terms of proliferation and activation the next step was to investigate whether IL-6 pre-exposure affected the cytokine production of naïve or memory CD4⁺ T-cells. Cytokine production by cells pre-exposed to IL-6 followed by 6-day stimulation with the low stimulus of 0.5µg/ml anti-CD3 and 1µg/ml anti-CD28 was measured by two approaches. These included quantification of cytokines found in the supernatants of naïve CD4⁺ T-cell cultures by MSD plates and analysis of intracellular cytokine production by naïve and memory CD4⁺ T-cells upon restimulation with PMA and ionomycin. The gating strategy for this latter approach employed the use of BFA only controls and can be seen in *Figure 6, Appendix*.

Firstly, MSD analysis of culture supernatants revealed substantial variation in cytokine production between experiments carried out with different donors, and none of the observed trends reached statistical significance. No clear relationship between IL-6 pre-exposure and cytokine production was observed, *Figure 32*.

Overall, there is no clear relationship between IL-6 pre-exposure and cytokine production as measured in the supernatants of stimulated naïve CD4⁺ T-cells.

Representative histograms from one donor for each cytokine detected by intracellular staining can be seen in *Figure 33*, which show the intensity of expression of each molecule and the number of cells with this intensity. This shows that there is no clear difference in expression of any cytokine in naïve CD4⁺ T-cells as 0.5ng/ml IL-6 pre-exposure.

Data from dot plots for 5 donors showing the percentage of cells positive for each cytokine was used to generate bar charts in *Figure 33*, representative dot plots can be seen in *Figure 18, Appendix*. These show that cytokines detected by intracellular staining amongst the memory (but not naïve) sub-population of CD4⁺ T-cells, did reveal a relationship between IL-6 pre-exposure and downstream cytokine production for IL-4 and IL-21 using non-parametric analysis of variance, $p < 0.05$, *Figure 33*. However, significance was not seen using a single Dunn's posthoc analyses for any cytokine with 0.5ng/ml IL-6 pre-exposure.

Dunn's posthoc analysis of all concentrations of IL-6 pre-exposed compared to non-exposed memory CD4⁺ T-cells revealed that for IL-4 and IL-21 only pre-exposure to supra-physiological concentrations of IL-6 (50ng/ml) caused significantly increased expression.

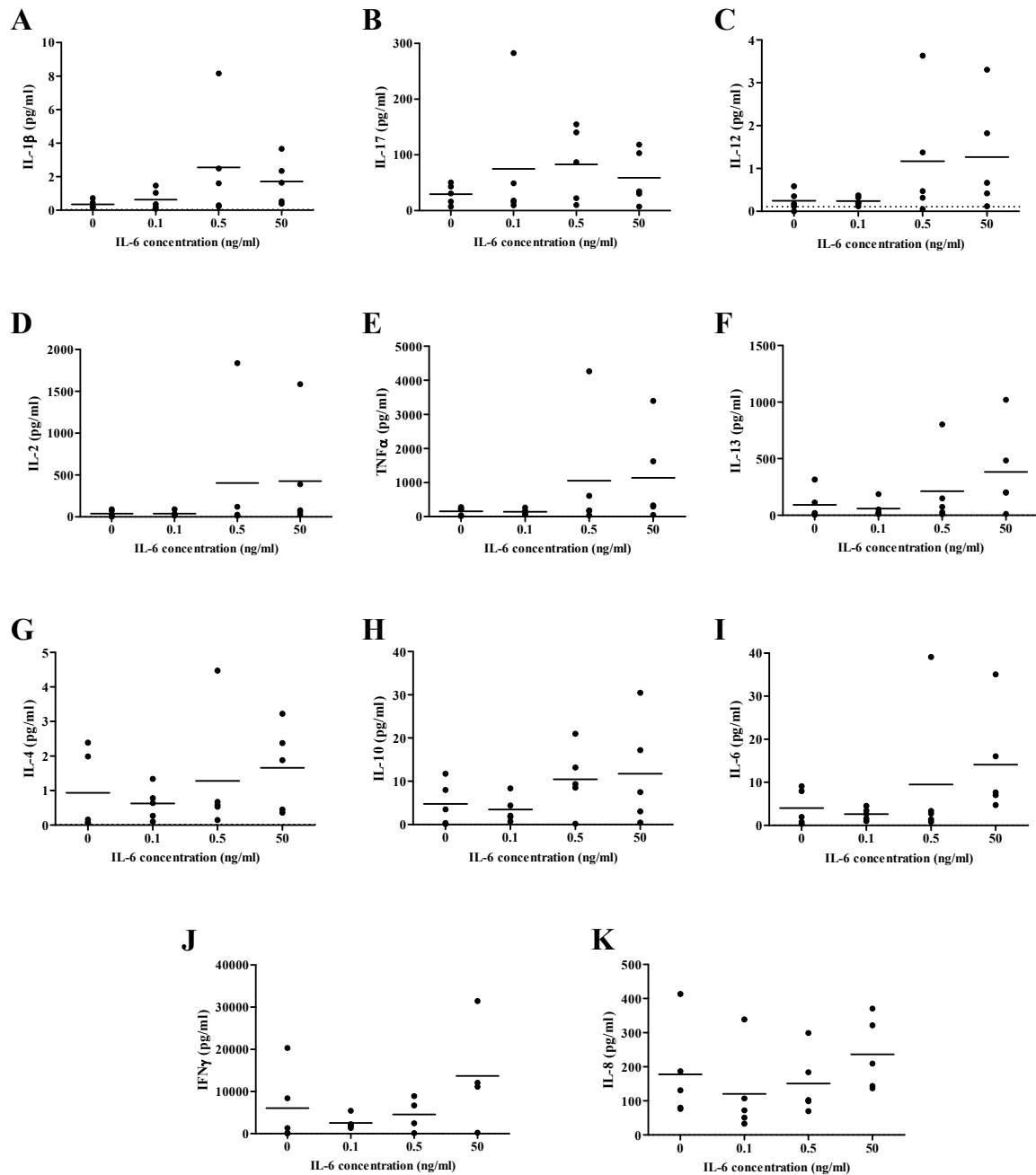


Figure 32. Cytokine production of naïve CD4⁺ T-cells following pre-exposure to IL-6 and subsequent TCR stimulation. Freshly isolated naïve CD4⁺ T-cells were cultured for 3 days in TexMACS alone, with 0.1ng/ml IL-6, 0.5ng/ml IL-6 or 50ng/ml IL-6 all with equimolar sIL-6R. Naïve CD4⁺ T-cells were washed and stimulated with 0.5 μ g/ml anti-CD3 and 1 μ g/ml anti-CD28 for 6 days. Following stimulation supernatants were harvested and cytokine levels measured by MSD including: IL-1 β (A), IL-17 (B), IL-12 (C), IL-2 (D), TNF- α (E), IL-13 (F), IL-4 (G), IL-10 (H), IL-6 (I), IFN- γ (J) and IL-8 (K). Values from 5 biological replicates were plotted with the horizontal line representing the median of all 5 experiments. The dotted line represents the detection threshold for the cytokine. Significance was determined by non-parametric analysis of variance (Friedman) with Dunn's posthoc pairwise analyses.

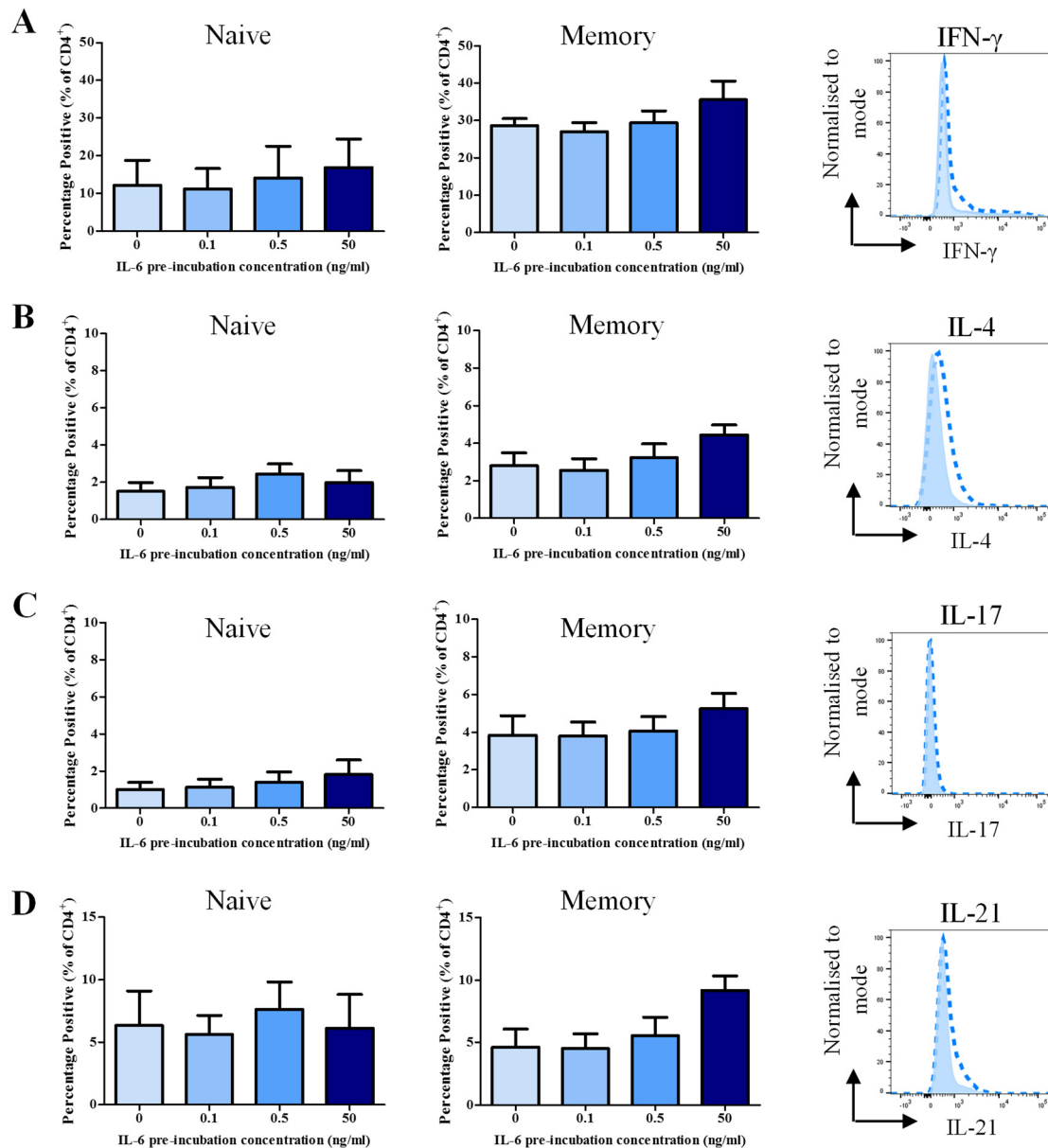


Figure 33. Cytokine production of naïve and memory CD4⁺ T-cells following pre-exposure to IL-6 and subsequent TCR stimulation. Freshly isolated naïve and memory CD4⁺ T-cells were cultured for 3 days in TexMACS alone, with 0.1ng/ml IL-6, 0.5ng/ml IL-6 or 50ng/ml IL-6 all with equimolar sIL-6R. After 3 days cells were washed and stimulated with 0.5 μ g/ml anti-CD3 and 1 μ g/ml anti-CD28 for a further 6 days. Following stimulation cells were rested overnight and stimulated with PMA/ionomycin followed by intracellular staining for flow cytometry. Cells were gated as singlets, lymphocytes and then CD4⁺ T-cells were identified as CD3⁺CD4⁺. Expression of IFN- γ (**A**), IL-4 (**B**), IL-17 (**C**) and IL-21 (**D**) were measured for 5 biological replicates as percentage positive using dot plots and values plotted with bar showing median and error bars representing SEM, the left panel shows naïve CD4⁺ T-cells and the middle panel shows memory CD4⁺ T-cells. The right panel shows representative histograms for untreated cells (pale blue shaded) and cells pre-exposed to 0.5ng/ml IL-6 (dark blue dashed line) showing intensities of expression of each cytokine. Non-parametric analysis of variance (Friedman) demonstrated a significant effect for IL-4 and IL-21 in memory CD4⁺ T-cells (* p <0.05). Comparisons of 0.5ng/ml IL-6 pre-exposed cells with non-pre-exposed cells were undertaken in a single Dunn's posthoc pairwise analysis.

To summarise, the wide variability in pro-inflammatory cytokine production observed in naïve CD4⁺ T-cells between biological replicates in these experiments hampers any confirmation of a relationship with IL-6 pre-exposure.

4.3.7 IL-6 pre-exposure influences T-helper cell differentiation

Due to the potential differences seen in cytokine production of memory CD4⁺ T-cells by intracellular staining, expression of transcription factors associated with effector differentiation was explored for association with IL-6 pre-exposure, *Figure 34*. Naïve and memory CD4⁺ T-cells were pre-exposed to IL-6 followed by 6-day stimulation with the low stimulus of 0.5µg/ml anti-CD3 and 1µg/ml anti-CD28 and transcription factors were measured by intracellular staining. The gating strategy for this analysis employed the use of unstained controls can be seen in *Figure 7, Appendix*.

Representative histograms from one donor for each transcription factor can be seen in *Figure 34*, which show the intensity of expression of each molecule and the number of cells with this intensity. This shows that there is no clear difference in expression of any transcription factor in naïve CD4⁺ T-cells with 0.5ng/ml IL-6 pre-exposure.

Data from dot plots for 5 donors showing the percentage of cells positive for each transcription factor was used to generate bar charts in *Figure 34*, representative dot plots can be seen in *Figure 19, Appendix*. These show that all transcription factors measured in memory CD4⁺ T-cells and all except ROR-γt in naïve CD4⁺ T-cells displayed significant increase in expression with increasing concentrations of IL-6 pre-exposure as determined by non-parametric analysis of variance, $p < 0.05$, *Figure 34*. In a single Dunn's posthoc analyses only GATA-3 in naïve CD4⁺ T-cells and Foxp3 in memory CD4⁺ T-cells displayed significantly increased expression following 0.5ng/ml IL-6 pre-exposure.

Dunn's posthoc analysis of all concentrations of IL-6 pre-exposed compared to non-exposed cells revealed that for all cytokines in memory CD4⁺ T-cells and for all cytokines except ROR-γt in naïve CD4⁺ T-cells pre-exposure to supra-physiological concentrations of IL-6 (50ng/ml) caused significantly increased expression.

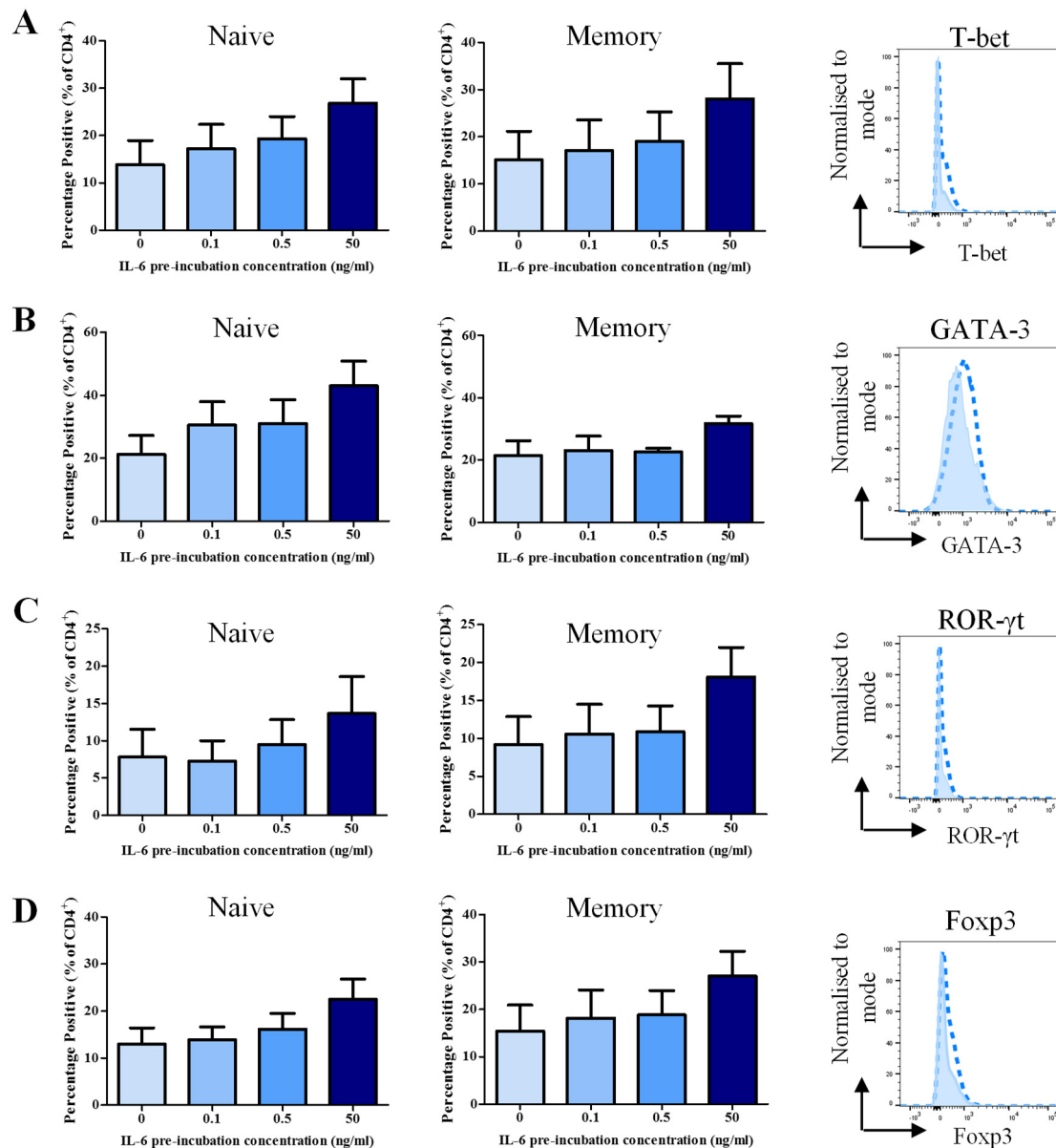


Figure 34. Naïve and memory CD4⁺ T-cell transcription factor expression following pre-exposure to IL-6 and subsequent TCR stimulation. Freshly isolated naïve and memory CD4⁺ T-cells were cultured for 3 days in TexMACS alone, with 0.1ng/ml, 0.5ng/ml or 50ng/ml IL-6, all with equimolar sIL-6R. After 3 days cells were washed and stimulated with 0.5μg/ml anti-CD3 and 1μg/ml anti-CD28 for a further 6 days. Following stimulation cells were rested overnight followed by intracellular staining for flow cytometry. Cells were gated as singlets, lymphocytes and then CD4⁺ T-cells were identified as CD3⁺CD4⁺. Expression of T-bet (A), GATA-3 (B), ROR-γt (C) and Foxp3 (D) were measured for 5 biological replicates as percentage positive using dot plots and values plotted with bars showing the median and error bars representing SEM, the left panel shows naïve CD4⁺ T-cells and the middle panel shows memory CD4⁺ T-cells. The right panel shows representative histograms for untreated cells (pale blue shaded) and cells pre-exposed to 0.5ng/ml IL-6 (dark blue dashed line) showing the intensity of expression of each transcription factor. Non-parametric analysis of variance (Friedman) demonstrated a significant effect for all transcription factors with the exception of ROR-γt in naïve CD4⁺ T-cells (*p<0.05 in each case). Comparisons of 0.5ng/ml IL-6 pre-exposed cells with non-pre-exposed cells were undertaken in a single Dunn's post hoc pairwise analysis *p<0.05.

Given the observed increase in transcription factors the possibility that pre-exposure to IL-6 may influence subsequent T-helper cell differentiation was explored. Naïve and memory CD4⁺ T-cells were cultured with 0.5ng/ml IL-6 and equimolar sIL-6R as optimised in the in vitro model, *Chapter 3.4.5*, followed by culture in their respective cytokine conditions, to induce differentiation of Th1, Th2, Th17 and Tfh. After 6 days of differentiation cells were rested before restimulation with PMA/ionomycin and stained for intracellular cytokines, employing an additional “BFA only” control for gating purposes, *Figure 20-23, Appendix*.

Differentiation of naïve and memory CD4⁺ T-cells towards their respective T-helper subsets was confirmed by comparison of cytokine expression of these cells compared to control stimulated undifferentiated naïve, memory or whole CD4⁺ T-cells, *Figure 35*. Representative plots can be seen in *Figures 20-23, Appendix*. Naïve CD4⁺ T-cells differentiated towards a Th1 phenotype had significantly increased production of IFN- γ , the key cytokine produced by Th1 cells, compared to naïve CD4⁺ T-cells cultured without the addition of Th1 skewing cytokines. Cells skewed towards Th1 had no difference in production of IL-4 or IL-17 however production of IL21 was significantly increased compared to non-skewed cells, *Figure 35A*. Naïve CD4⁺ T-cells differentiated towards Th2 cells showed no significant differences in cytokine production. The lack of increase in IL-4 suggests differentiation towards Th2 was not successfully achieved, *Figure 35B*. Memory CD4⁺ T-cells differentiated towards Th17 displayed significantly reduced production of IFN- γ and increased production of both IL-17 and IL-21 of cells compared to non-skewed memory CD4⁺ T-cells, confirming the differentiation of this cell subset, *Figure 35C*. In contrast, CD4⁺ T-cells differentiated towards Tfh cell phenotype showed only a slight increase in IL-21 production which did not achieve statistical significance, and no difference in IFN- γ , IL-4 or IL-17 production, *Figure 35D*.

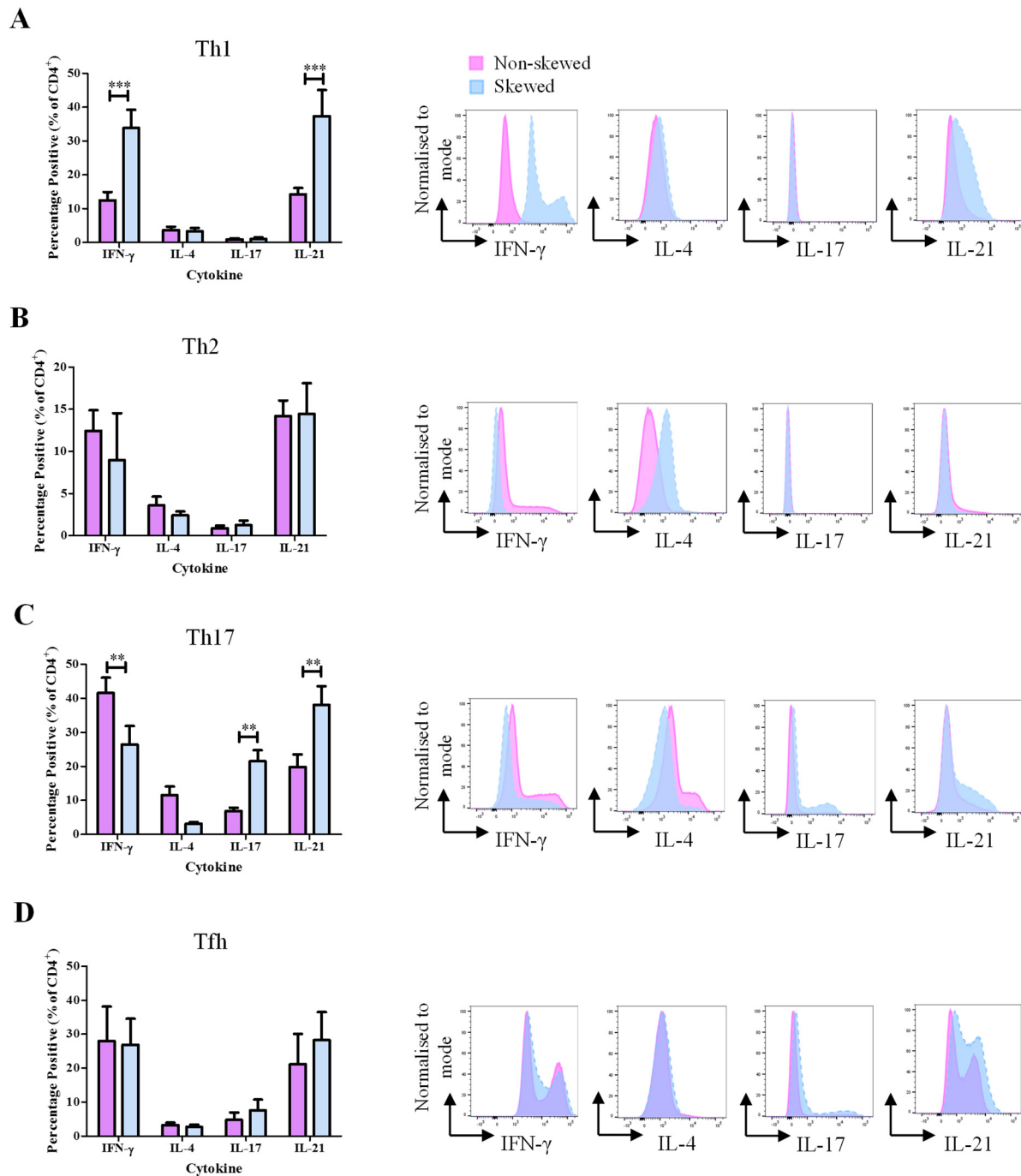


Figure 35. Cytokine production of CD4⁺ T-cells with or without differentiation towards T-helper cell subsets. Freshly isolated total CD4⁺ T-cells, naïve CD4⁺ T-cells or memory CD4⁺ T-cells were cultured for 3 days in TexMACS, IMDM +10% FCS or IMDM + SR respectively. After 3 days cells were washed and differentiated towards Th1 (A), Th2 (B), Th17 (C) or Tfh (D) for 6 days. Following differentiation cells were rested for 1-4 days before stimulation with PMA/ionomycin and staining for intracellular cytokines for flow cytometry. Cells were gated as singlets, lymphocytes and then CD4⁺ T-cells were identified as CD3⁺CD4⁺. Representative histograms of cytokine expression shown for non-skewed cells (pink) and cells skewed towards T-helper subsets (blue) shows intensities of expression of each cytokine. Values for 6 biological replicates were determined using percentage positive dot plots and were plotted with bar showing median and error bars representing SEM. Significance was determined by two-way ANOVA and Bonferroni posthoc analyses ** p<0.05, ***p<0.001.

Next, naïve, memory or whole CD4⁺ T-cells were cultured in their respective differentiation cocktails following pre-exposure to 0.5ng/ml IL-6 and equimolar sIL-6R. Naïve CD4⁺ T-cells differentiated towards a Th1 phenotype had no difference in production of IL-4, IL-17 or IL-21 but displayed increased ability to produce IFN- γ following pre-exposure to 0.5ng/ml IL-6 compared to cells not exposed to IL-6, *Figure 36A*. Naïve CD4⁺ T-cells differentiated towards Th2 cells had no difference in cytokine production following exposure of cells to 0.5ng/ml IL-6 compared to non-pre-exposed cells. Although decreased IFN- γ and IL-21 levels were seen with IL-6 pre-exposure of Th2 cells this, did not reach significance, *Figure 36B*. Moreover, memory CD4⁺ T-cells pre-exposed to 0.5ng/ml IL-6 prior to their differentiation into Th17 cells exhibited no difference in IFN- γ , IL-4 or IL-21 production but they did exhibit significantly decreased production of IL-17 compared to memory CD4⁺ T-cells not exposed to IL-6 prior to differentiation, *Figure 36C*. Finally, CD4⁺ T-cells pre-exposed to 0.5ng/ml IL-6 prior to differentiation towards Tfh cells had no difference in IFN- γ , IL-4 or IL-17 production, however these cells demonstrated a decrease in the production of IL-21 compared to non-pre-exposed Tfh cells, *Figure 36D*.

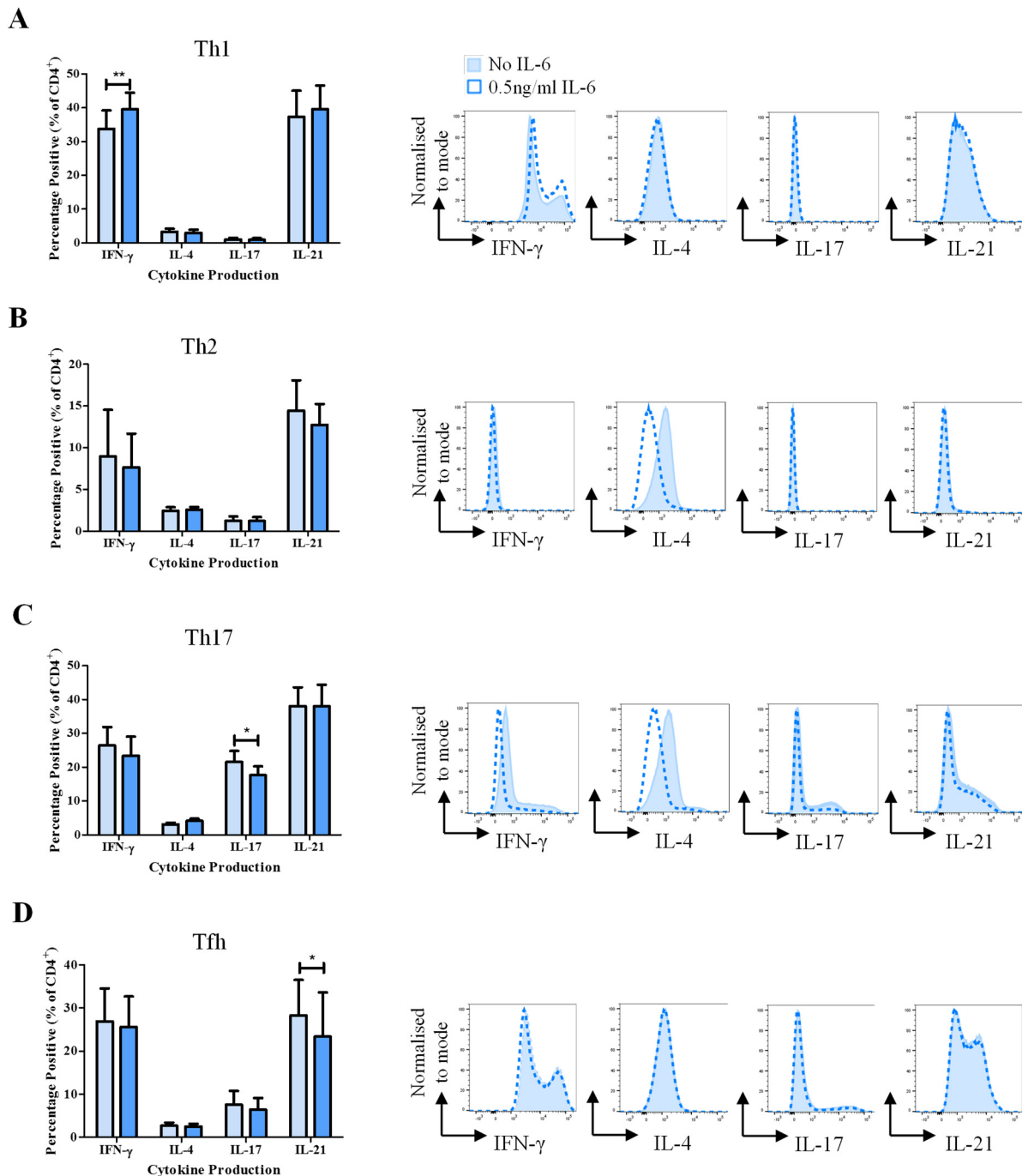


Figure 36. Cytokine production of T-helper cells differentiated following pre-exposure to IL-6. Freshly isolated total CD4⁺ T-cells, naïve CD4⁺ T-cells or memory CD4⁺ T-cells were cultured for 3 days in TexMACS, IMDM +10% FCS or IMDM + SR respectively, alone or with 0.5ng/ml IL-6 with equimolar sIL-6R. After 3 days cells were washed and differentiated towards Th1 (A), Th2 (B), Th17 (C) or Tfh (D) for 6 days. Following differentiation cells were rested for 1-4 days before stimulation with PMA/ionomycin and staining for intracellular cytokines for flow cytometry. Cells were gated as singlets, lymphocytes and then CD4⁺ T-cells were identified as CD3⁺CD4⁺. Representative histograms of cytokine expression shown for untreated cells (pale blue shaded) and cells pre-exposed to 0.5ng/ml IL-6 (dark blue dashed line) show intensities of expression of each cytokine. Values from 6 biological replicates were determined using percentage positive dot plots and were plotted with bar showing median and error bars representing SEM. Significance was determined by two-way ANOVA and Bonferroni post hoc analyses; * p<0.05, **p<0.01.

To summarise the findings from this section, pre-exposure to IL-6 resulted in increased expression levels of the major transcription factors involved in T-helper cell differentiation in both naïve and memory CD4⁺ T-cells. Pre-exposure of naïve or memory CD4⁺ T-cells to IL-6 does not affect the differentiation of either Th2 or Tfh cells but, as depicted in summary form in *Figure 37*, has the ability to influence Th1 and Th17 cell differentiation, which could have consequences on further function in the immune setting.

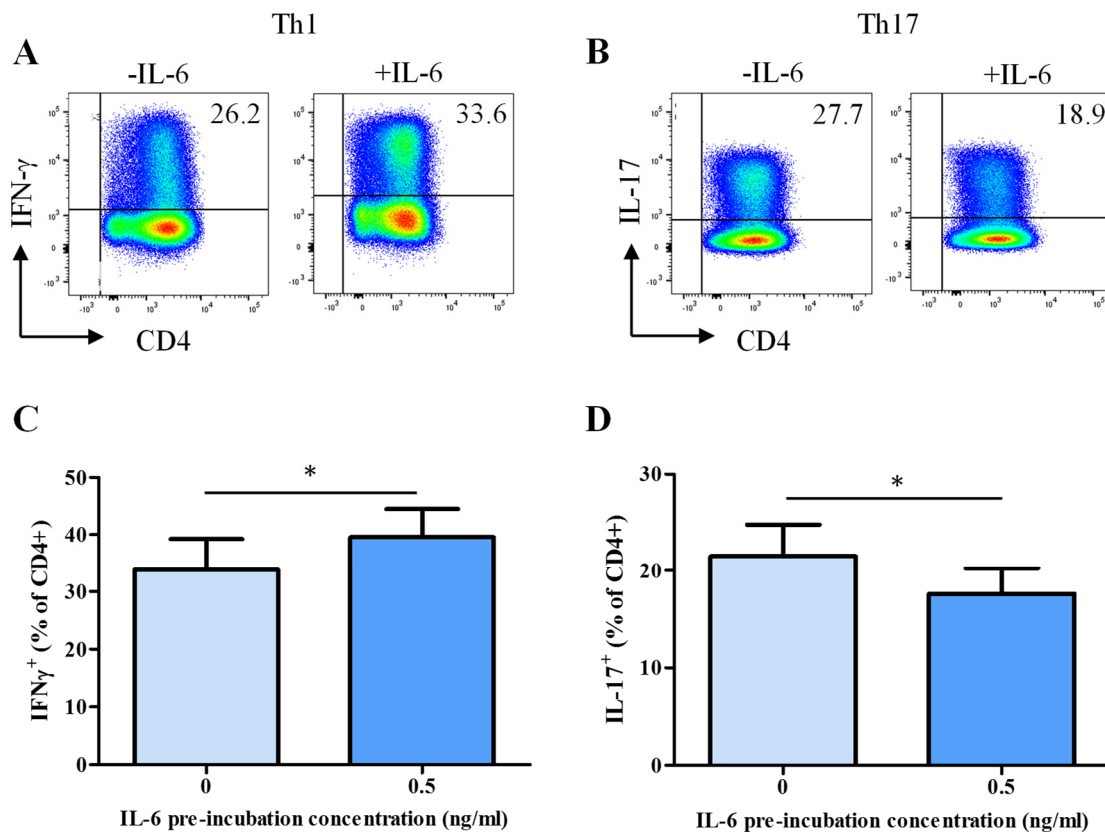


Figure 37. Figure summarising main findings from Figure 36 in relation to effects of IL-6 pre-exposure upon T-cell differentiation. Freshly isolated naïve CD4⁺ T-cells or memory CD4⁺ T-cells were cultured for 3 days in IMDM + 10% FCS or 10% SR respectively, alone or with 0.5ng/ml IL-6 with equimolar sIL-6R. After 3 days cells were washed and differentiated towards Th1 (A and C), Th17 (B and D) for 6 days. Following differentiation cells were rested for 1-4 days before being stimulated with PMA/ionomycin and stained for intracellular cytokines for flow cytometry. Cells were gated as singlets, lymphocytes and then CD4⁺ T-cells were identified as CD3⁺CD4⁺. Values from 6 biological replicates were determined using percentage positive dot plots (A and B) and were plotted with bar showing the median and error bars representing SEM (C and D). Significance was determined by Wilcoxon matched-pairs signed rank test * p<0.05.

4.3.8 Cytokine analysis of culture supernatants

IL-6 could affect T-cell effector function and phenotype either directly or indirectly through the induction of other factors. Many mediators were examined by MSD multiplex during IL-6 pre-exposure. One factor IL-6 can induce is IL-2, which is of interest as this is a T-cell growth factor, which may be responsible for the effects seen with IL-6 pre-exposure. In order to rule out the effects observed are directly a consequence of IL-6, levels of IL-2 were measured in the culture supernatants during the 3 day IL-6 pre-exposure period. From this, it can be seen that the level of IL-2 in culture supernatants with 0.5ng/ml IL-6 was minimal and reflected the level of IL-2 in the untreated naïve CD4⁺ T-cells and the levels of IL-2 remained stable over the 3 days of culture, *Figure 38*. Levels of IL-2 were substantially higher in the 50ng/ml IL-6 cultured naïve CD4⁺ T-cells over the 3 days of culture which may contribute to the altered effector function seen with this high concentration of IL-6. This suggests IL-2 is induced in response to supra-physiological levels of IL-6 exposure, however, at lower physiological concentrations seen in RA, there is no increased induction of IL-2 compared to untreated samples. This suggests that the effects on T-cells seen with 0.5ng/ml IL-6 are not indirectly due to enhanced IL-2 induction.

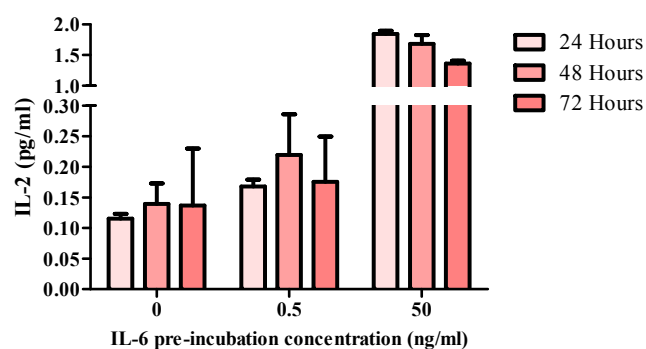


Figure 38. Concentration of IL-2 in the supernatants of naïve CD4⁺ T-cells during 3 days pre-exposure to IL-6. Naïve CD4⁺ T-cells were cultured for 3 days with TexMACS alone, with 0.5ng/ml IL-6 or with 50ng/ml IL-6 with equimolar sIL-6R. Supernatants were harvested at 24 hours, 48 hours and 72 hours of culture and subject to MSD analysis. Values from 3 biological replicates were plotted with bar representing mean and error bars representing SEM.

4.4 Discussion

In this chapter, the aim was to establish whether chronic pre-exposure of naïve and memory CD4⁺ T-cells to IL-6 could influence the function of effector CD4⁺ T-cells following TCR stimulation, by employing an in vitro system in which IL-6 was excluded at the point of TCR stimulation. The focus was to evaluate the consequences of IL-6 pre-exposure on CD4⁺ T-cell proliferation, activation, viability, cytokine production and T-helper differentiation. The effects of IL-6 on these parameters are summarised in *Table 14*. The current investigation is, to my

knowledge, the first to examine the degree to which long-lasting prior exposure of naïve CD4⁺ T-cells to IL-6 augments effector function downstream of subsequent TCR stimulation.

Phenotypic consequences of IL-6 pre-exposure	Naïve CD4⁺ T-cells	Memory CD4⁺ T-cells
Viability	No effect	No effect
Proliferation	Increased	Increased
Activation	Increased CD25 and CD40L	No effect
Cytokines	No effect	No effect
Transcription Factors	Increased GATA-3	Increased Foxp3
T-helper differentiations	Increased IFN- γ in Th1	Decreased IL-17 in Th17

Table 14. Overall effects of IL-6 pre-exposure on naïve and memory CD4⁺ T-cells. IL-6 pre-exposure has no effects on viability or cytokine production however does increase cell proliferation and activation with effects on Th1 and Th17 differentiation.

The difference in response to IL-6 pre-exposure between naïve and memory CD4⁺ T-cells may be a result of the different expression of IL-6 receptors of these two subsets. The difference in response may reflect the functional characteristics of these cell subsets as memory CD4⁺ T-cells are required for cytokine production and rapid response against antigen whereas naïve CD4⁺ T-cells generally do not produce cytokines and have not encountered cognate antigen in the periphery (475).

4.4.1 Induction of STAT-3 target genes

The 12-gene signature described in *Chapter 1.3.12*, which can identify a subset of RA patients, contains a significant number of STAT-3 inducible genes (431). STAT-3 is classically induced via ligation of the gp130 co-receptor. A number of IL-6 family cytokines members signal in this way to induce STAT-3, including IL-11, LIF, OSM, CNTF, CT-1, CLC, IL-27 and IL-31. In addition, STAT3 is also induced by other cytokines via alternative signalling mechanisms including IFNs, IL-2, IL-12, IL-10, IL-23, IL-21, G-CSF and leptin.

In previously published work, several cytokines such as IL-4, IL-10, IL-17 and IL-23 as well as other serum mediators of STAT-3 (G-CSF, leptin, IL-6R), were measured in the serum of early RA patients but found to be undetectable and/or unrelated to STAT-3 target gene expression. Furthermore, only IL-6 (and not the alternate pro-inflammatory cytokine TNF) was able to independently predict expression of the STAT-3 genes (431).

Of the 3 genes explored in this chapter one of the genes, BCL-3 had no induction upon stimulation with any pro-inflammatory or STAT-3 cytokines. This may be due to a difference in time kinetics of this gene. Work carried out in the lab indicates that while maximal induction of SOCS3 occurs with 6 hours of cytokine stimulation the maximal induction of BCL-3 varies between 6-16 hours (Natasha West, Newcastle University). Therefore, further investigation into the time-kinetics of BCL-3 induction is required. Of the IL-6 family/STAT-3 utilising cytokines tested in the current experiments, only IL-21 was able to induce the STAT-3 target genes to a similar extent as IL-6. IL-21 signals via IL-21R to induce JAK1 and JAK3 and, similarly to IL-6, subsequently to induce STAT-1 and STAT-3 with the balance of these determining cell functions (476). Like IL-6, IL-21 contributes to the inhibition of IFN- γ and Th1 differentiation but enhances Th17 and Tfh cells, although this is not the only cytokine necessary for their development (163, 477, 478). IL-6 has been shown to induce the production of IL-21 by CD4⁺ T-cells, which is necessary for indirect promotion of Ig production (298, 385). It is possible that circulating IL-21 may contribute to the STAT-3 signature described in early RA (479, 480); reliable measurement of the cytokine in serum has been attempted by our research group in the past but proved technically difficult, and this should be revisited in the light of these findings.

4.4.2 Consequences of IL-6 pre-exposure on downstream effector function

4.4.2.1 Proliferation

The effect of IL-6 pre-exposure on proliferative capacity following stimulation was most evident in the naïve CD4⁺ T-cell subset. The presence of IL-6 has been heavily implicated in T-cell proliferation responses in previous studies. In animal models the deletion of IL-6R results in an impaired ability of T-cells to proliferate (481). In contrast, the excessive signalling of IL-6 in mice drives accelerated CD4⁺ T-cell proliferation (403).

Specifically, utilisation of the trans-signalling pathway by IL-6 has been shown to promote T-cell proliferation in a number of contexts utilising animal models of disease, such as during colon cancer, in a TGF- β dependent manner (482). Further studies have also implicated the trans signalling pathway as playing a key role in the proliferation of hepatocytes and regulation of liver regeneration (483, 484). However, the in vitro finding that IL-6 pre-exposure effects upon downstream proliferative capacity does not require the presence of sIL-6R suggests the effect of this mechanism is not dependent on trans signalling in human cells. Despite this, the current work cannot be said to definitively link the observation to classical signalling alone. For example, it is possible that IL-6R shedding by CD4⁺ T-cells in response to classical IL-6 signalling occurs, in turn permitting trans-signalling in the in vitro system.

For this study, both a high level of TCR stimulation and a lower “sub-optimal” level of stimulus were used. This was because a high level of stimulation may result in complete proliferation of all cells meaning an effect of IL-6 pre-exposure would be masked. The sub-optimal stimulus indeed proved more reliable for identifying dose effects in relation to IL-6 pre-exposure. Furthermore, the impurities seen in the naïve and memory CD4⁺ T-cell populations due to the isolation method chosen must be taken into account. Naïve CD4⁺ T-cells had a purity of >85% and so it could be possible that the contaminating cells could be playing a role in increased levels of proliferation seen. Equally memory CD4⁺ T-cells were typically only >60% pure with a high level of contamination from naïve CD4⁺ T-cells, this could suggest that in the memory population the small proportion of naïve CD4⁺ T-cells may be resulting in the effects seen.

4.4.2.2 Activation and signalling receptors

Phenotypic markers CD27, CD28, CD25 and CD40L were chosen in order to assess the expression of co-stimulatory molecules and markers of early CD4⁺ T-cell activation. CXCR5 was chosen as a marker of Tfh cells, a subset which IL-6 has been heavily implicated in the development and LAP was also measured as a marker of TGF- β production for information on regulatory properties.

It has previously been described that upon antigen exposure in vitro there is a progressive loss of CD28 followed also by the loss of CD27 (459). This is reflected in this Chapter as expression of CD28 of naïve CD4⁺ T-cells is decreased from baseline 6 days stimulation, *Chapter 4.3.5*. In contrast, the expression of CD27 on naïve CD4⁺ T-cells remains at a high level post stimulation. Based on the literature CD27 would also be expected to decrease following stimulation. One reason for its stability may be that loss of CD28 is described to occur before loss of CD27 so the time point sampled post-TCR stimulation may be too early to notice any change in expression. Alternatively, it may be due to the type and strength of stimulation. Stimulation of these cells was via anti-CD3 and anti-CD28 with no antigen present, it is possible that antigen exposure or the cytokine milieu produced by APCs is required for decreased CD27 expression.

Similarly, expression of IL-6R was shown to be down regulated following TCR stimulation, whereas the expression of gp130 appears to be upregulated following TCR stimulation, *Chapter 4.3.5*. The down regulation of IL-6R upon stimulation is confirmed by other studies which report infiltrating effector cells shed the IL-6R upon stimulation via the TCR, correlating with increased sIL-6R levels (367, 368). The up-regulation of gp130 has been documented in smooth muscle cells upon stimulation with viral IL-6, a homologue of IL-6, or with IL-6 and sIL-6R in an autocrine stimulation loop, however, this has not been reported in CD4⁺ T-cells upon TCR

stimulation (231, 485). One explanation for its rapid upregulation could be the pleiotropic nature of this molecule means it is used by many different cytokines and its rapid turnover may reflect the necessity of this molecule in other signalling pathways following activation.

Upon TCR stimulation of IL-6 pre-exposed CD4⁺ T-cells there is an increased expression of the activation markers CD25 and CD40L; as with the finding of increased proliferative capacity, the finding is most prominent in the naïve CD4⁺ T-cell sub-population. Following the discovery of IL-6, this cytokine described as a T-cell co-stimulatory molecule due to its ability to enhance proliferation when present alongside TCR stimulation, rather than as a “pre-programming” mediator as used in this study (486). This effect on proliferation is well characterised as independent of IL-2, however, here increased expression of CD25, the IL-2R α , on naïve CD4⁺ T-cells suggests a possible role for IL-2. IL-2 is both produced and consumed by CD4⁺ T-cells and is a crucial cytokine in T-cell survival and proliferation (487). The increased expression of CD25 permits increased signalling via IL-2 as this molecule functions to increase the affinity of the IL-2R for its ligand (488). This increased level of IL-2 signalling may result in altered effector function. It is also possible that increased expression of CD25 acts to sequester IL-2 and limit its availability to Tregs, as has been described in Tregs following antigen dependent activation (489). Furthermore, CD8⁺ T-cells expressing high levels of CD25 appear to proliferate more rapidly with a more pronounced effector phenotype (490). Due to this IL-2 has been measured in the supernatants of cultured T-cells to detect the importance of this cytokine in the effects seen as a result of IL-6 pre-exposure, *Chapter 3.3 8*.

CD40L expression was increased following IL-6 pre-exposure and the ligation of this molecule with its receptor CD40 on DCs has been shown to result in the production of IL-6, potentially resulting in the creation of a positive feedback loop (491). The interaction of CD40-CD40L has also been shown to be critical for Th17 differentiation in vitro and in vivo as well as influencing the polarisation of Th1 and Th2 cells shown to play a role in pathogenesis of EAE and CIA (491, 492).

CXCR5 is a marker present of Tfh cells, whose development is thought to require IL-6. Although IL-6 pre-exposure did not result in increased expression of this marker after 6 days stimulation literature has found IL-6 stimulation results in early and rapid upregulation of CXCR5 (493). Here, it was reportedly seen within the first 24 hours following antigen exposure so it may be possible that more of a difference would be apparent if phenotyping had been carried out at this earlier time point.

LAP is a membrane-bound form of TGF- β 1, which is a marker of suppressive function. This molecule has been seen to be decreased in the presence of IL-6 in a STAT-3 dependent manner, by inhibition of Lrrc32, the glycoprotein that binds TGF- β to the membrane (494). In contrast, LAP has been reported to be strongly increased upon strong TCR stimulation (Amy Anderson, Newcastle University, unpublished data). Despite these findings, no difference in LAP expression was seen according to IL-6 pre-exposure and levels of this marker remained low throughout the 6 days of stimulation.

Studies have indicated that both the presence of IL-6 alone or the presence of IL-6 with simultaneous TCR stimulation can mediate CD4⁺ T-cell survival by maintaining BCL-2 expression and downregulating FasL to protect from AICD (472, 495, 496). Furthermore, mice with deficient IL-6 signalling have reduced numbers of T-cells (497), although others have found that IL-6R α KO have no difference in apoptosis rates (481). Despite the documented role of IL-6 in cell survival in this study the pre-exposure of CD4⁺ T-cells to IL-6 had no effect on cell viability following stimulation. It may be that the effects of IL-6 on cell survival is an immediate effect and cannot be maintained when IL-6 is no longer present in the culture.

4.4.2.3 Cytokines and differentiation

It was also seen that the pre-exposure of naïve and memory CD4⁺ T-cells to IL-6 resulted in increased transcription factor expression (GATA-3 and FoxP3) and may alter cytokine production specifically in memory CD4⁺ T-cells following TCR stimulation, *Chapter 4.3.7*. Altered effector phenotypes were also observed under Th1 and Th17 skewing conditions, *Chapter 4.3.8*.

IL-6 pre-exposure resulted in the increased expression of GATA-3 in naïve CD4⁺ T-cells and the increased expression of Foxp3 in memory CD4⁺ T-cells. IL-6 pre-exposure resulted in significant dose-dependent increase in transcription factor expression with increasing concentrations of IL-6 pre-exposure as determined by non-parametric analysis of variance, indicating that resting cells are more activated towards their pre-programmed phenotype when pre-exposed to IL-6. This effect is strongest in memory CD4⁺ T-cells which have already undergone activation and subsequent differentiation in vivo whereas naïve CD4⁺ T-cells have not undergone this process. Another possibility is that the increased expression of these markers with IL-6 pre-exposure is a result of increased activation and proliferation of these cells, as more cells are activated following pre-exposure to IL-6 there is a higher presence of these transcription factors. IL-6 has been shown to induce ROR- γ t in mice, however, this requires the presence of TGF- β (498). The pre-exposure of cells to IL-6 also shows the ability to induce ROR- γ t, however, this effect is specific to memory CD4⁺ T-cells.

The increased expression of transcription factors is also reflected in the increased cytokine production of memory CD4⁺ T-cells following IL-6 pre-exposure. In memory CD4⁺ T-cells pre-exposed to IL-6 there is significant dose-dependent increase in IL-4 and IL-21 expression with increasing concentrations of IL-6 pre-exposure as determined by non-parametric analysis of variance, which correspond to increased GATA-3, ROR- γ t and with the increased CXCR5 representing Tfh cells. In contrast, naïve CD4⁺ T-cells have no difference in cytokine production following IL-6 pre-exposure, however, this may be due to the fact that naïve CD4⁺ T-cells do not readily produce cytokines whereas memory CD4⁺ T-cells need to rapidly respond to antigen mediated activation with cytokine production in order to generate the immune response necessary to eliminate pathogens.

When present at the time of stimulation IL-6 is known to influence the differentiation and hence cytokine production of CD4⁺ T-cells. Under these circumstances IL-6 has been shown to promote both IL-4 and IL-21 production in a STAT-3 dependent manner (361, 385, 499). The pre-exposure of memory CD4⁺ T-cells to IL-6 shows the increased production of IL-21 and IL-4. Conversely, presence of IL-6 during stimulation has been shown to inhibit IFN- γ production by inducing SOCS1, however, with IL-6 pre-exposure there was in fact an increase in IFN- γ .

Due to the increased expression of transcription factors and cytokine production by cells pre-exposed to IL-6 it was then explored whether cells pre-exposed to IL-6 prior to differentiation may result in altered cytokine production. This is the case of Th1 cells which display increased IFN- γ production and Th17 cells which show decreased IL-17 production.

RA has historically been categorised as a Th1 mediated disease with IFN- γ being the dominant cytokine implicated. However, there has been no clear evidence for increased IFN- γ production in patients with the disease. The increased production of IFN- γ by Th1 cells pre-exposed to IL-6 provides a mechanism of IFN- γ contribution to disease where levels although not particularly high result in a more pathogenic cell type and this could reflect the increased expression of T-bet following IL-6 pre-exposure. Despite the suggestion of RA as a Th1 mediated disease the targeting of IFN- γ has been shown to be unsuccessful in RA.

With the emergence of Th17 cells came the suggested theory that RA was an inflammatory disease mediated by IL-17, however, IL-17 is not obviously increased in RA and the targeting of this cytokine is unsuccessful in the treatment of RA. Despite the increase in ROR- γ t with IL-6 pre-exposure there was decreased IL-17 production of Th17 cells pre-exposed to IL-6 prior to differentiation. In mice, IL-2 has been shown to inhibit the development of Th17 cells and as previously mentioned the increased expression of CD25 by IL-6 pre-exposed cells suggests

a role for this cytokine in potential pathogenic effects. Although this has been studied in mice it is possible that IL-2 also inhibits the development of human Th17 cells reflected in the decreased production of IL-17 seen with IL-6 pre-exposure.

Despite no firm role for either subset in disease pathogenesis it is clear that the balance of these cells is pertinent to disease progression and this balance may be altered by the pre-exposure of CD4⁺ T-cells to IL-6. These conflicting stories although do suggest a T-cell specific role in disease and other T-cell mediators may be important.

4.4.3 The effect of IL-2 in the IL-6 pre-exposure model

In order to assess whether the effects of IL-6 pre-exposure seen on CD4⁺ T-cell phenotype were due to the direct effects of IL-6 or were due to an indirect effect via the induction of other mediators, cell culture supernatants of IL-6 exposed cells were analysed for the presence of cytokines which may impact downstream effector function. IL-6 is able to induce the production of IL-2 which is a key cytokine in the survival of CD4⁺ T-cells (500). It is possible that the effects of IL-6 pre-exposure on effector phenotype is due to the increased production of IL-2 by these cells which in turn has consequences on cell phenotype. However, it was seen that in culture supernatants of cells exposed to 0.5ng/ml IL-6 compared to untreated cells there was no significant increase in IL-2 levels in the culture at any time point over the 3 day culture. At the 50ng/ml concentration of IL-6 the levels of IL-2 are greatly increased. However, this is a supra-physiological concentration of IL-6 whereas more physiological concentrations appear to have no effect. This suggests that IL-2 is not responsible for the effects seen on naïve CD4⁺ T-cells following IL-6 pre-exposure.

4.4.4 Summary

In summary, here it is shown that pre-exposure to increasing concentrations of IL-6 and equimolar sIL-6R prior to TCR stimulation resulted in increased levels of proliferation and increased expression of activation markers and transcription factors in naïve and memory CD4⁺ T-cells. Although there was no clear effect of IL-6 pre-exposure on naïve CD4⁺ T-cell cytokine production following TCR stimulation, memory CD4⁺ T-cells display possible alterations in cytokine production. Furthermore, this priming event does appear to impact the cytokine production of cells differentiated towards Th1 and Th17 phenotypes, apparently favouring the differentiation of Th1 effector cells. Despite the numerous effects of IL-6 pre-exposure the effects on proliferation of naïve and memory CD4⁺ T-cells appears to be lost following shorter periods of IL-6 pre-exposure suggests these pathogenic effects require chronic stimulation with IL-6 and are not the result of transient IL-6 exposure. The findings suggest the possibility that “cytokine-programming” of CD4⁺ T-cells in the circulation may occur, whereby dysregulated

effector function becomes “imprinted” even when the subsequent antigen encounter/ TCR activation occurs in the absence of the cytokine in question.

Chapter 5. Effects of IL-6 pre-exposure on naïve and memory CD4⁺ T-cell gene expression

5.1 Introduction

One of the key objectives of the work outlined in this thesis was to investigate the molecular consequences of IL-6 pre-exposure on naïve and memory CD4⁺ T-cells. Given the clear differences in effector phenotype of CD4⁺ T-cells pre-exposed to IL-6 it was next important to investigate whether IL-6 pre-exposure had an effect on the transcriptional profile of these cells, an effect which will ultimately cause dysregulated effector function. Gene expression analysis is now commonplace and there is a wealth of CD4⁺ T-cell expression data available from numerous experimental contexts. However, the specific question of cytokine pre-exposure has not yet been addressed.

IL-6 has been shown to affect the expression of a number of genes in CD4⁺ T-cells, such as the transcription factors BCL-6, c-Maf, GATA-3 and STAT-3 (298, 501-503). Others have also shown the ability of IL-6 to induce both IFN- γ and IL-4 mRNA expression (364, 474). Deinz et al. have explored the gene expression of mouse CD4⁺ T-cells at early time-points following TCR-stimulation in the presence of a supra-physiological concentration of 100ng/ml IL-6 (385). This reveals differential expression of 132 genes, 16 hours following TCR-stimulation. The most striking induction is IL-21 with over 200-fold induction. Other notably induced genes included ROR- γ/α , ARID5b, ID2, BCL-3, CCR5, IL-4R α , SOCS3 whereas a number of genes were also downregulated including CD4, IL-7R, STAT-4, FasL and RANKL.

Furthermore, in RA patients that exhibit good clinical responses to anti IL-6R antibody tocilizumab there is a significantly reduced expression of ARID5a (504). This molecule has also been shown to govern the fate of naïve CD4⁺ T-cell effector function. IL-6 induces the expression of this molecule in mice CD4⁺ T-cells during Th17 differentiation in a STAT-3 dependent manner.

This work outlined in this Chapter will explore whether pre-exposure of naïve and/or memory CD4⁺ T-cells results in an upregulation of these previously defined IL-6 induced genes and what other genes are mediated by the presence of IL-6 but also pre-exposure of cells to IL-6 followed by TCR-stimulation when IL-6 is no longer present in an effort to probe the situation in early RA.

Naïve and memory CD4⁺ T-cells are both phenotypically and functionally distinct cell subsets and this is reflected in their difference in response to IL-6 pre-exposure, *Chapter 4*. Despite the

huge differences in naïve and memory phenotype and function these cell types are highly similar at the level of gene expression with 95% of genes overlapping (505). The differences in expression programmes largely reflect differences in effector function, migration and homeostasis. Differentially expressed genes between naïve and memory cells have been shown to exhibit enrichment of those harboured by risk loci for immune-mediated diseases generally implicating memory CD4⁺ T-cells in disease pathogenesis (506).

The purpose of the work carried out in this Chapter was to test the hypothesis that IL-6 pre-priming alters downstream transcriptional profiles, specifically in naïve CD4⁺ T-cells as these cells appear most sensitive to IL-6 at the level of STAT-3 signalling, *Chapter 1.3.12*. Gene expression analysis carried out in IL-6 pre-exposed naïve and memory CD4⁺ T-cells, in parallel, will ascertain whether effects of IL-6 pre-exposure are more pronounced in the naïve sub-population.

5.2 Aims and objectives

In the previous chapters, an in vitro model to mimic chronic IL-6 pre-exposure was established and the consequences of this pre-exposure on naïve and memory CD4⁺ T-cell effector function determined. Following IL-6 pre-exposure naïve and memory CD4⁺ T-cells displayed increased cell proliferation and activation and altered cytokine production of memory CD4⁺ T-cells, in addition to altered cytokine profiles following T-helper cell differentiation. Employing the same in vitro model but utilising fresh blood from healthy donors rather than LRS cones, the overall aim of the work carried out in this chapter was to investigate the dynamic molecular consequences of pro-longed IL-6 pre-exposure on naïve and memory CD4⁺ T-cells, both before and after TCR stimulation.

The specific objectives were:

- To determine the transcriptional consequences of IL-6 exposure amongst naïve and memory CD4⁺ T-cells over a 72 hour period, and the extent to which these account for previously observed CD4⁺ T-cell deregulation in early RA. In doing so this will assess the relative transcriptional sensitivity of naïve and memory CD4⁺ T-cells to IL-6.
- To determine the genes differentially induced during T-cell receptor stimulation, or differentially expressed 4 hours after stimulation, according to prior exposure to IL-6.
- To examine the molecular pathways induced by IL-6 which may explain the differences in effector cell phenotype following IL-6 pre-exposure, described in *Chapter 4*.

5.3 Results

5.3.1 Design of dynamic CD4⁺ T-cell transcriptome experiment

To investigate the effects of IL-6 pre-exposure on the naïve and memory CD4⁺ T-cell transcriptome five healthy donors were recruited to derive cellular material for use in this experiment, with ages ranging between 24-40 and female to male ratio of 2:3, details can be seen in *Table 3, Appendix*. In each case, 120mls blood was drawn into citrate vacutainers. Naïve and memory CD4⁺ T-cells were isolated from fresh whole blood and the purity of these cells, determined using gating on whole CD4⁺ T-cells, *Figure 2, Appendix*, can be seen in *Table 15*. Purity of naïve CD4⁺ T-cells is consistently high however purity of memory CD4⁺ T-cells are much lower, with the majority of contaminants being CD45RA⁺ CD4⁺ T-cells, therefore caution should be taken when interpreting gene expression data for memory CD4⁺ T-cells.

Donor	Purity of naïve CD4 ⁺ T-cells (CD45RA ⁺ % of alive lymphocytes)	Purity of memory CD4 ⁺ T-cells (CD45RO ⁺ % of alive lymphocytes)
1	89.3	80.9
2	95.7	67.7
3	93.7	65.4
4	95.7	54.9

Table 15. Purity of naïve and memory CD4⁺ T-cells used for subsequent microarray analysis. Purity was calculated for each healthy donor as the percentage of CD45RA or CD45RO in the alive lymphocyte population.

Following isolation naïve or memory CD4⁺ T-cells were then cultured for 3 days with 0.5ng/ml IL-6 and equimolar sIL-6R and during this period, RNA was extracted 6 hours and 72 hours post-IL-6 exposure. The concentration of 0.5ng/ml IL-6 used in the transcriptional profiling of CD4⁺ T-cells was chosen as this was deemed to be both physiologically relevant and cause substantial consequences on CD4⁺ T-cell effector function, as seen in *Chapter 4*, in terms of proliferation, activation and T-helper cell differentiation.

As in the in vitro model, cells were thoroughly washed before stimulation with 0.2µg/ml anti-CD3 and 1µg/ml anti-CD28, optimised for citrate whole blood. Post-TCR stimulation RNA was extracted at 4 hours, 16 hours and 6 days. The time points chosen to take RNA for profiling were chosen in order to capture both early and late responses in gene expression as a result of

IL-6 exposure and TCR stimulation. The induction of STAT-3 regulated genes was found to be induced by IL-6 following 6 hours cytokine stimulation (*Figure 24, Chapter 4.3.1*) therefore this time point should reveal genes directly induced as a result of IL-6 stimulation. Following 72 hour exposure to IL-6 and prior to TCR stimulation RNA was taken as this provides a baseline to assess which genes are deregulated by IL-6 at the point directly prior to stimulation. Time points post-TCR stimulation were 4 hours and 16 hours in order to capture both early and late changes in gene expression. The 6-day time point was also chosen to address whether there was altered gene expression at the time of altered effector phenotype and whether there were any long lasting consequences on gene expression of IL-6 pre-exposure. The experimental design is summarised in *Figure 39*.

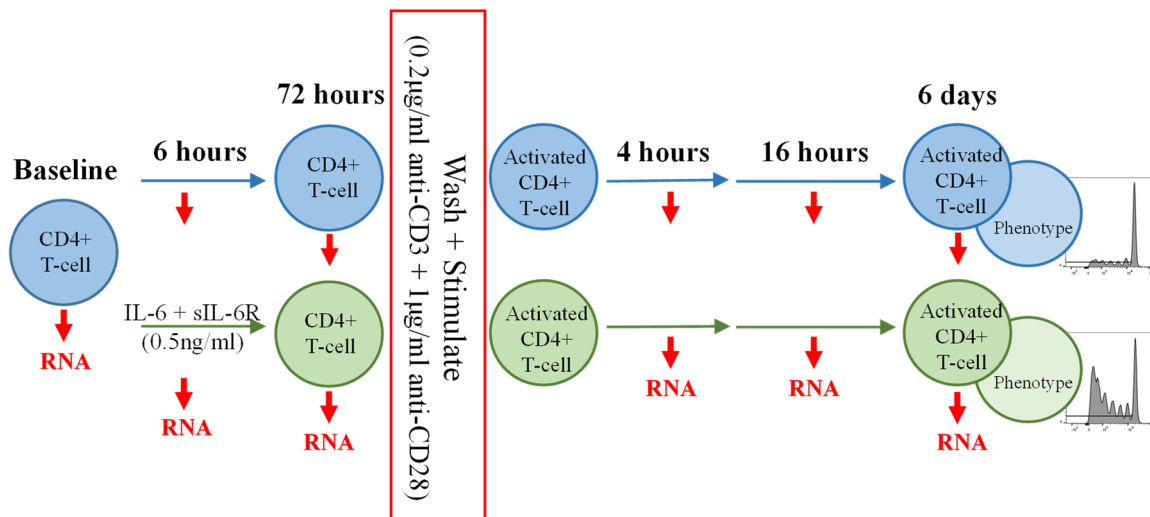


Figure 39. IL-6 Pre-exposure model for dynamic transcriptome experiment. Naïve and memory CD4⁺ T-cells were isolated from healthy donor blood drawn into citrate and were cultured for 3 days in TexMACS alone or with 0.5ng/ml IL-6 and equimolar sIL-6R. . After 3 days cells were washed and stimulated with 0.2µg/ml anti-CD3 and 1µg/ml anti-CD28 for a further 6 days. RNA was taken at baseline, after 6 hours and 72 hours IL-6 exposure and 4 hours, 16 hours and 6 days post-TCR stimulation. RNA was processed into double stranded cDNA, amplified to create cRNA and hybridised to Illumina Human HT-12 v4.0 microarray for genome expression profiling. In parallel, an aliquot of CFSE labelled naïve CD4⁺ T-cells were cultured as described and following 6 days stimulation acquired on the flow cytometer to analyse proliferation.

In order to ensure if inference about any relationship between dynamic gene expression and effector phenotype amongst CD4⁺ T-cells in this experiment was valid, the proliferative capacity of naïve and memory cells from each donor was assessed using residual cells. With the exception of donor 5, all donors appeared to show clear increases in proliferation between the untreated and 0.5ng/ml IL-6 pre-exposed naïve CD4⁺ T-cells, *Figure 40A*. As donor five did not appear to respond to TCR-stimulation nor to IL-6 pre-exposure this donor was excluded from the experiment resulting in 4 donors which showed good responses to IL-6 pre-exposure with fold induction between 2-8%, *Figure 40B*. RNA was extracted from these donors and microarray analysis was performed.

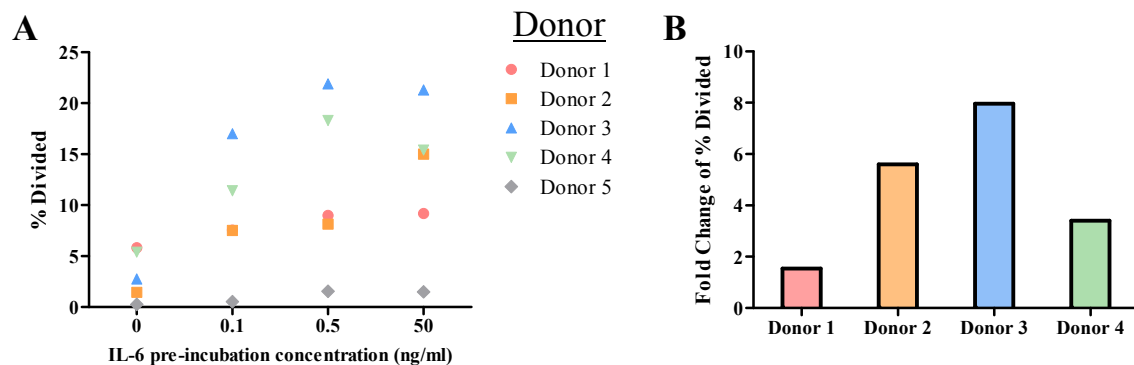


Figure 40. Proliferation of naïve CD4⁺ T-cells from healthy donors recruited for microarray. Freshly isolated naïve CD4⁺ T-cells from blood drawn into citrate were CFSE labelled and cultured for 3 days in TexMACS alone, with 0.1ng/ml IL-6, 0.5ng/ml IL-6 or 50ng/ml IL-6 all with equimolar sIL-6R. After 3 days cells were washed and stimulated with 0.2µg/ml anti-CD3 and 1µg/ml anti-CD28 for a further 6 days. Following stimulation cells were acquired on the flow cytometer. Proliferation was calculated using FlowJo with percentage divided indicating the precursor frequency of dividing cells (**A**) and fold change determined by the level of proliferation at 0.5ng/ml compared to the untreated condition (**B**).

5.3.2 Quality control of microarray data and pairwise analysis strategy

Prior to performing differential gene analysis, data quality was assessed in R using the Bioconductor package (445, 507). An overview of the bioinformatics performed are outlined in *Chapter 2.12*. Briefly, box plots of raw data from all 4 donors were used to assess background intensities of all arrays, *Figure 41A*. Data was normalised both within array and between arrays as described by VST and RSN and box plots of normalised data were assessed, *Figure 41B* (446). Following normalisation all samples were deemed to be of good quality and were taken forward for further differential expression analysis.

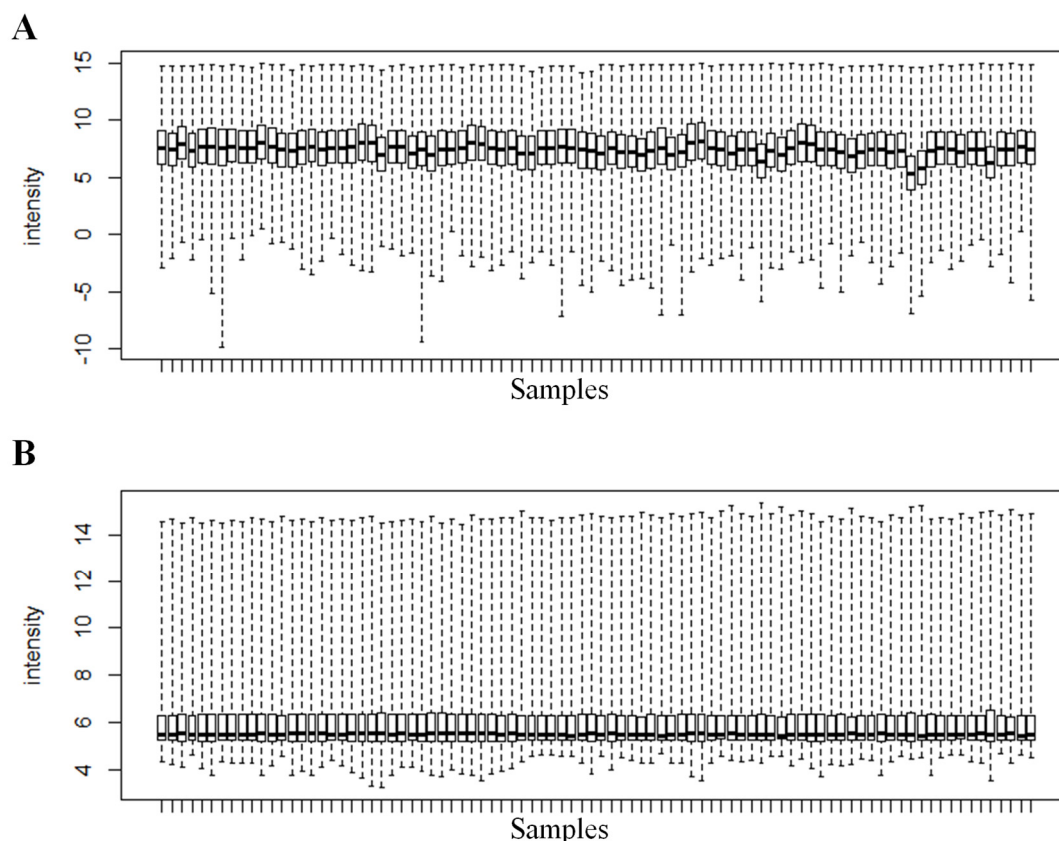


Figure 41. Boxplots showing intensities of all samples on microarray. Data quality was assessed in R using the Bioconductor package. Background intensities of all samples from 4 donors were visualised as boxplots prior to normalisation (A) or following VST and RSN normalisation (B).

As all arrays passed this quality control stage, principle component analysis plots were generated which plot the measures that account for the largest amount of variance in the data set. Upon inspection of the PCA it was revealed that one of the donors, donor 4, appeared not to have responded to TCR stimulation, as all samples from this donor cluster around the baseline, 6-hour and 72-hour time pre-TCR stimulation time points of the other 3 donors, *Figure 42*. The difference in gene expression of this donor is difficult to explain and it could not be definitively excluded that it may have resulted from experimental error as opposed to inter-

individual variability. For these reasons, samples from this donor have been excluded from downstream analyses. Naïve and memory CD4⁺ T-cells from the other 3 donors reveal that cells appear to cluster together based on time-point. Pre-stimulation samples cluster together and post-stimulation samples also cluster according to time point but also remain distinct from each other, *Figure 42*.

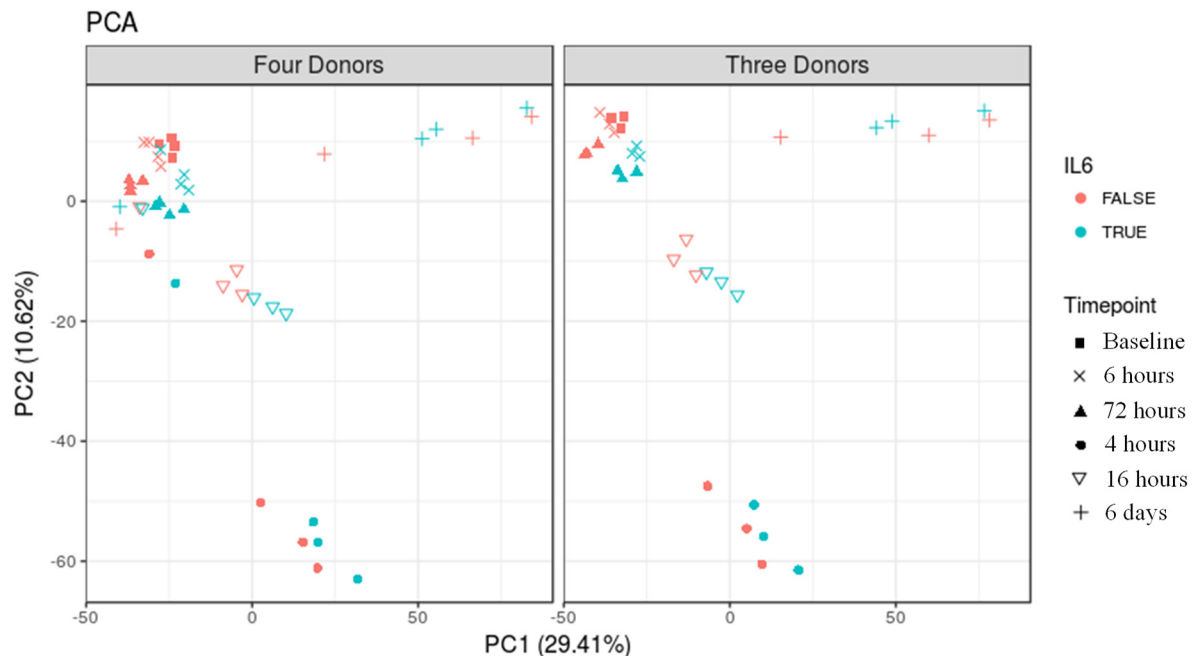


Figure 42. Principle component analysis of non-pre-exposed or IL-6 pre-exposed naïve CD4⁺ T-cells. Principle component analysis was carried out on gene expression data from the Illumina Human HT-12 v4.0 expression BeadChip array using the Bioconductor package in R software. The left hand plot represents the data with all 4 healthy donors included and the right hand plot represents the data following removal of donor number 4. Symbols represent the different time-points throughout culture with IL-6 and following TCR stimulation. Untreated naïve CD4⁺ T-cells are coloured red and IL-6 pre-exposed naïve CD4⁺ T-cells are coloured blue.

The limma package (450) was used for differential gene expression analysis. This package uses a linear model where standard variances are moderated across genes allowing the shrinking of individual gene variance towards a pooled estimate; this allows for more stable inference where sample size is limited (508). The analysis also allowed multi-level comparisons to combine paired comparisons and comparisons between two independent groups, ultimately allowing for the variation between donors. In addition, around 30% of probes are non-specific and have sequences which match to more than one gene. Probes which can match to more than one gene can result in a doubling of signal intensity and cause false positive results. For this reason, these probes were removed from the analysis. Further analyses involved cross-sectional differential

expression to identify genes differentially expressed at each time point in IL-6 pre-exposed cells compared to untreated cells and longitudinal differential induction analyses to identify genes differentially induced following TCR stimulation in IL-6 pre-exposed cells compared to untreated cells. These analyses are summarised in *Figure 43*.

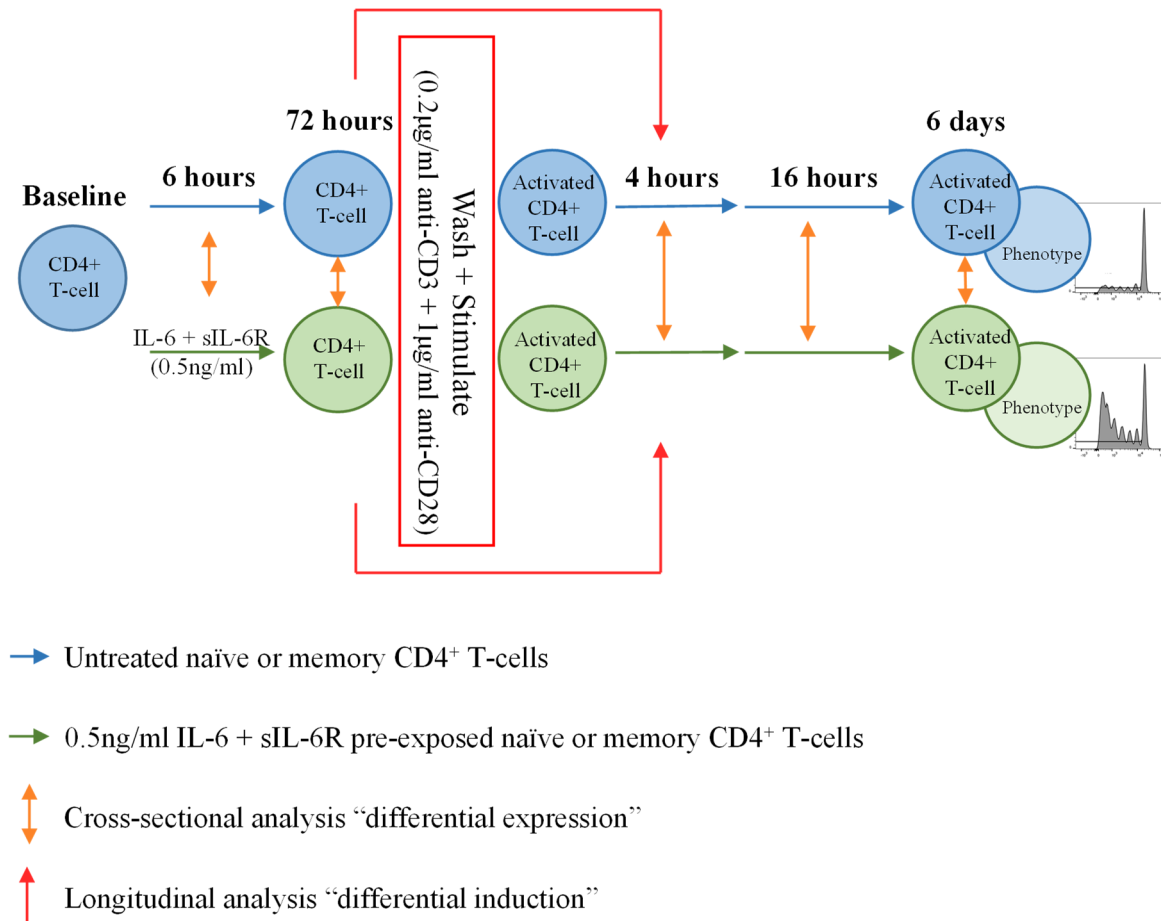


Figure 43. Analyses conducted on the dynamic transcriptome experiment. Naïve or memory CD4⁺ T-cells were cultured alone (blue arrows) or with 0.5ng/ml IL-6 and equimolar sIL-6R (green arrows) followed by stimulation with 0.2µg/ml anti-CD3 and 1µg/ml anti-CD28. RNA was taken at time-points indicated. Cross-sectional analysis was performed at each time point to identify genes differentially expressed between untreated and IL-6 pre-exposed naïve and memory CD4⁺ T-cells (orange arrows). Longitudinal analysis was performed to identify genes differentially induced following TCR stimulation as a result of IL-6 pre-exposure (red arrows).

5.3.3 Effect of IL-6 pre-exposure on naïve and memory CD4⁺ T-cell gene expression prior to TCR stimulation

The first analysis carried out was a cross section analysis to identify genes differentially expressed between the IL-6 exposed and non-exposed CD4⁺ T-cells amongst the three donors who passed quality control at the “6 hour post-IL-6” time-point in the dynamic transcriptome experiment described above in *Chapter 5.3.2*. Results were compared in naïve and memory

CD4⁺ T-cell subsets. Differentially expressed genes were identified using an adjusted p-value cut off of <0.05 and a log fold change (LFC) of 1.5. Genes were subject to multiple test correction using the Benjamini-Hochberg method (451). Differentially expressed genes are depicted as volcano plots in *Figure 44A-B*. Volcano plots are a visual representation of genes on the microarray, those that reach statistical significance in pairwise comparison of differentially expressed genes, according to their FC and corrected p-value thresholds, appear as red dots. The absolute numbers of differentially expressed genes (DEGs) identified in this way are displayed in *Table 16*, which also summarises the same information at other time-points in the experiment. Corresponding gene lists can be found in the CD-ROM in the *Appendix*.

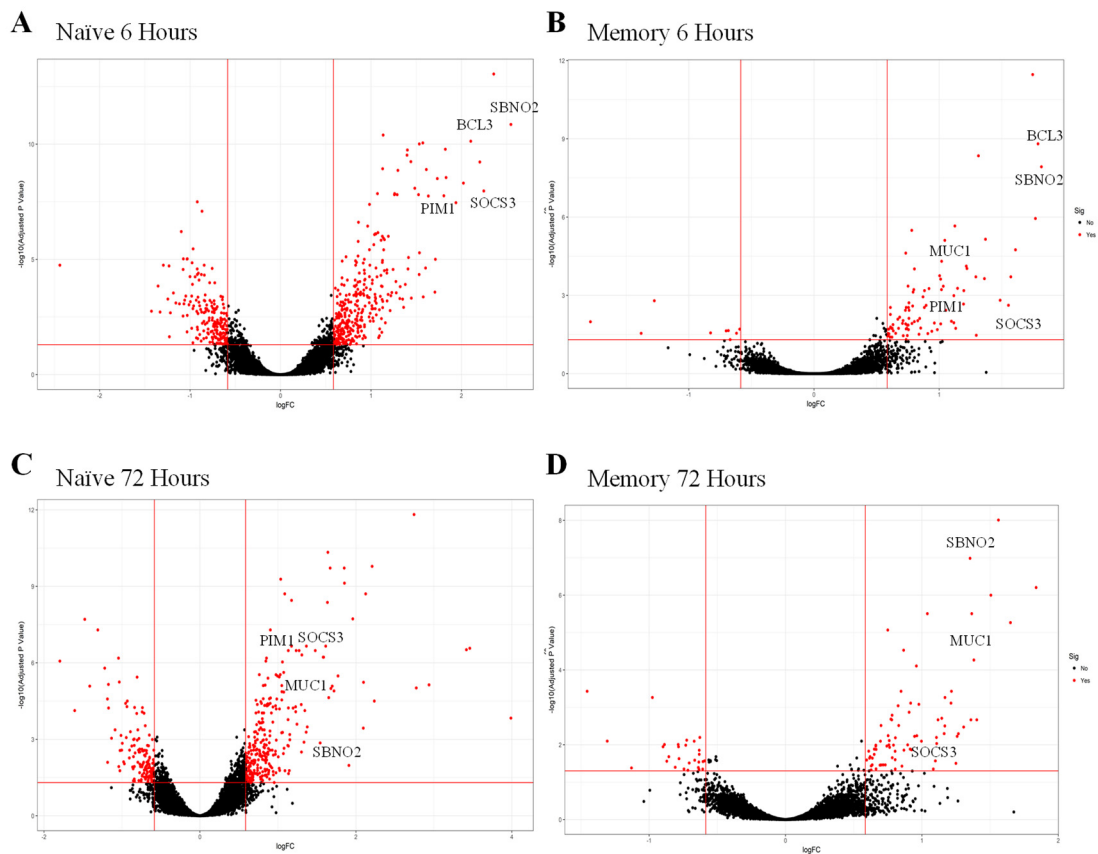


Figure 44. Volcano plots showing differentially expressed genes between untreated and IL-6 exposed naïve and memory CD4⁺ T-cells following 6 hours and 72 hours IL-6 exposure. Differentially expressed genes were identified post multiple test correction by cross sectional analysis of untreated and IL-6 exposed naïve (**A** and **C**) and memory (**B** and **D**) CD4⁺ T-cells from 3 biological replicates following 6 hours and 72 hours exposure to 0.5ng/ml IL-6 using paired t-test with log fold change 1.5 and corrected p value cut off of <0.05. STAT-3 regulated genes found to be dysregulated in early RA are highlighted.

Time-point	Naïve CD4 ⁺ T-cells	Memory CD4 ⁺ T-cells
6 hours post-IL-6	532	99
72 hours post-IL-6	428	104
4 hours post-TCR	233	10
16 hours post-TCR	8	0
6 days post-TCR	0	0

Table 16. Number of differentially expressed genes between untreated and IL-6 pre-exposed naïve and memory CD4⁺ T-cells at each time point. Differentially expressed genes were identified by cross sectional analysis of untreated and IL-6 exposed naïve and memory CD4⁺ T-cells from 3 biological replicates at each time point using paired t test with log fold change 1.5 and corrected p value cut off of <0.05.

Exposure of naïve CD4⁺ T-cells to IL-6 and sIL-6R altered the expression of 532 genes by <1.5-fold at 6 hours (adjusted p<0.05), with only 99 genes similarly modulated amongst the mature memory CD4⁺ T-cell counterparts, *Figure 44, CD-ROM Gene lists 1A and 2A*. This pattern was maintained at 72 hours with 428 and 104 genes in naïve and memory CD4⁺ T-cells, respectively, *Figure 44, CD-ROM Gene lists 1B and 2B*. This pattern was also present at 4 hours following IL-6 removal and TCR stimulation with 233 versus 10 genes and again at 16 hours post-TCR stimulation with differentially expressed genes only occurring in naïve CD4⁺ T-cells, *Table 16, Gene lists 1C, 2C and 1D*. The transcripts which were impacted in memory CD4⁺ T-cells corresponded for the most part to a subset of those impacted in naïve CD4⁺ T-cells, with very few genes differentially expressed in memory CD4⁺ T-cells alone at each time-point, *Figure 45*. These data confirmed, at the transcriptional level, that naïve CD4⁺ T-cells are markedly more sensitive to sustained IL-6 exposure.

Figure 45. The overlap of differentially expressed genes between untreated and IL-6 pre-exposed naïve and memory CD4⁺ T-cells at each time point. Venn diagrams indicate the differentially expressed genes identified by cross sectional analysis of untreated and IL-6 exposed naïve and memory CD4⁺ T-cells from 3 biological replicates at each time point using paired t test with log fold change 1.5 and corrected p value cut off of <0.05. N=3.

confirms that naïve and memory CD4⁺ T-cells have been exposed to IL-6 in the in vitro model, with a significant dose to induce changes in gene expression.

To explore the extent to which the IL-6 responsive genes observed in this Chapter corresponded to those we previously observed to be differentially expressed in total CD4⁺ T-cells of early RA patients compared to disease controls (431), DEGs at the 6 hour time-point amongst naïve and memory CD4⁺ T-cells in the current experiment were contrasted with the published dataset. A number of the DEGs identified were STAT-3 regulated genes, found to be components of a 12-gene “signature” previously shown to robustly discriminate early RA from disease controls. These included BCL3, PIM1, SOCS3, SBNO2 and, uniquely in memory CD4⁺ T-cells, MUC1, *Figure 44A and B*. Furthermore, of an extended list of 96 genes found to be differentially expressed between early RA and non-RA controls before multiple test correction (see *CD-ROM Supplementary Gene List* (431)) 30 were also found to be differentially expressed in the IL-6 exposed naïve CD4⁺ T-cells and 15 were found to be differentially expressed in IL-6 exposed memory CD4⁺ T-cells in the current study. The 15 genes found to be differentially expressed in memory CD4⁺ T-cells were the same as found in naïve CD4⁺ T-cells with the exception of MUC1 and SNORA10.

Hypergeometric testing was then carried out on these gene lists which is a statistical test to determine the probability of successes (genes differentially expressed between early RA and disease controls) from the sample population (IL-6 exposed naïve or memory CD4⁺ T-cells). This level of overlap was significantly higher than would be expected by chance in the memory CD4⁺ T-cells with hypergeometric testing resulting in p-values of 2.22E-15. A smaller number of differentially expressed genes between untreated and IL-6 treated memory CD4⁺ T-cells overlap with DEGs found in early RA, however, this still remains significantly higher than would be expected by chance suggesting that although memory CD4⁺ T-cells are less sensitive to the effects of IL-6 they also contribute to the gene signature seen in early RA.

5.3.4 CD4⁺ T-cell gene expression changes pre-TCR stimulation are partially maintained over 72 hours

It was of interest to explore the extent to which dysregulation of genes identified after 6 hours IL-6 exposure was sustained after 72 hours of culture in this experimental set up, modelled, as it was to mimic “chronic” exposure to the cytokine. A pair-wise analysis of DEGs at 72 hours was therefore undertaken, analogous to that described above.

The number of DEGs identified after 72 hours IL-6 exposure were 428 genes and 104 genes in naïve and memory CD4⁺ T-cells, respectively, *Table 16*. At this 72 hour time point differential

induction of STAT-3 regulated genes between untreated and IL-6 pre-exposed naïve CD4⁺ T-cells are largely maintained from 6 hours including PIM1, SOCS3, SBNO2 and MUC1 which is now found in both naïve and memory CD4⁺ T-cells, *Figure 44C*. Although naïve CD4⁺ T-cells gain differential expression of MUC1, they appear to lose differential expression of BCL3 at this time point, despite this gene having a log fold change over 1.5 the adjusted p value does not reach significance. On the other hand, the memory CD4⁺ T-cells only maintain differential expression of MUC1, SOCS3 and SBNO2, *Figure 44D*.

Again, in previously published data by Pratt et. al. of the 96 DEGs found between early RA and non-RA patients, 18 of these genes were found to be differentially expressed as a result of IL-6 pre-exposure in naïve CD4⁺ T-cells compared to 11 of these genes found to be differentially expressed by IL-6 pre-exposure in memory CD4⁺ T-cells at 72 hours. Once more, this level of overlap is significantly higher than would be expected by chance with hypergeometric testing resulting in p-values of 9.59E-09 for naïve CD4⁺ T-cells and 8.99E-10 for memory CD4⁺ T-cells. The dynamic profiles of genes significantly dysregulated at both 6 hours and 72 hours in naïve CD4⁺ T-cells can be seen in *Figure 46*, with genes previously observed to be differentially expressed in early RA indicated. Despite a number of DEGs in memory CD4⁺ T-cells as a result of 72 hours IL-6 exposure overlapping with genes identified by Pratt et al as differentially expressed in early RA, a higher number of DEGs in naïve CD4⁺ T-cells as a result of 72 hours IL-6 exposure overlap with these previously identified DEGs in early RA, further suggesting that naïve CD4⁺ T-cells are the crucial cell type responding to IL-6 signalling.

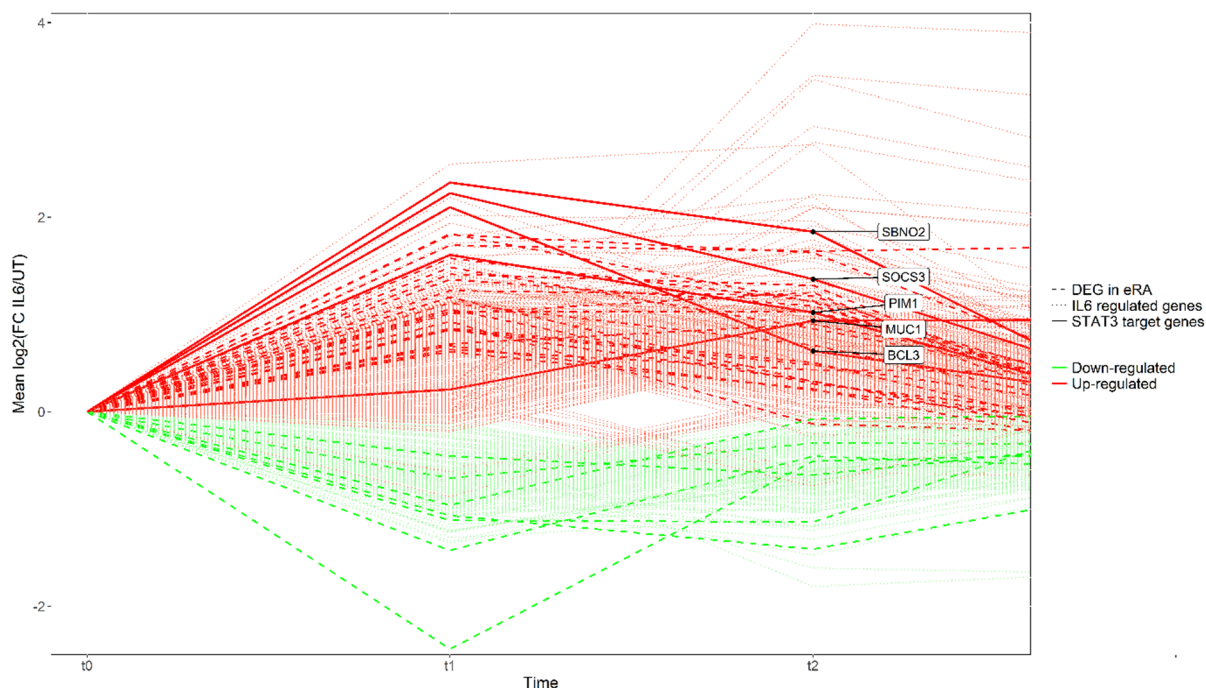


Figure 46. Dynamic profiles of differentially expressed genes between untreated and IL-6 exposed naïve CD4⁺ T-cells following 6 hours or 72 hours. Fold-expression in IL-6 exposed cells is depicted relative to that in non-exposed cells at 6 hours (t1) and 72 hours (t2) with log 2 fold change >1.5 and corrected p<0.05 for inclusion. Genes previously observed to be differentially expressed (DE) in early RA (eRA) are indicated (heavy lines), and specific STAT-3 targets labelled (solid lines).

The top 10 differentially expressed genes following 72 hours exposure to IL-6 in naïve CD4⁺ T-cells are summarised in *Table 17*. Of these genes 5 were chosen for validation and further investigation in *Chapter 6* to explore whether these genes were differentially expressed in disease conditions. The induction of these genes, termed the “IL-6 pre-TCR signature”, was assessed in naïve CD4⁺ T-cells following 72 hours exposure to IL-6 by real-time PCR, *Figure 47*. All genes showed increased expression following IL-6 treatment compared to untreated cells and this increase reached significance for BCL-6, SBNO2 and REXO2. Full gene lists can be seen in *CD-ROM Gene List 1B and 2B*.

Gene Symbol	Illumina ID	Log Fold Change	P-value
BCL6*	ILMN_1737314	2.745663468	3.385E-13
REXO2*	ILMN_1749009	1.035768851	7.174E-10
AF038185	ILMN_1833858	1.640052332	7.506E-10
LRP8*	ILMN_1724533	1.669138016	7.506E-10
PRG4	ILMN_1780349	2.125162982	8.759E-10
FAM113B	ILMN_1712431	1.634692162	2.46E-09
ID2	ILMN_1793990	1.959497127	2.057E-08
SBNO2*	ILMN_1808811	1.850223353	6.21E-08
C10orf10*	ILMN_1767556	1.612132122	1.404E-07
SULT1B1	ILMN_1733443	1.306667282	4.41E-07

Table 17. Top 10 differentially expressed genes in naïve CD4⁺ T-cells following 72 hours IL-6 exposure. Genes shown are the top 10 differentially expressed genes with the strongest p values with their respective log fold changes and Illumina IDs. Genes highlighted with * represent those genes used for further validation of the IL-6 pre-TCR signature in *Chapter 6*.

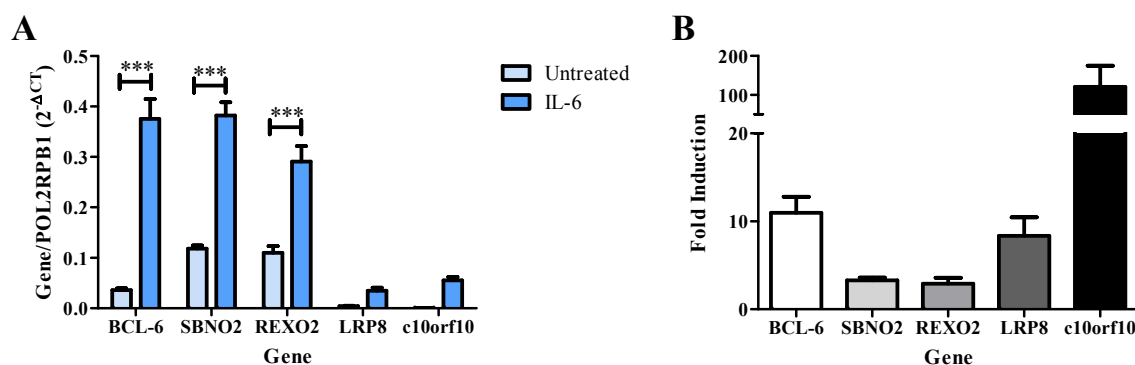


Figure 47. Validation of “IL-6 pre-TCR signature” genes in naïve CD4⁺ T-cells following 72 hours IL-6 exposure. Naïve CD4⁺ T-cells from 5 healthy donors were cultured with 0.5ng/ml IL-6 and equimolar sIL-6R for 72 hours and RNA was extracted. 5 genes shown to be differentially expressed following 72 hours IL-6 exposure were assessed by real-time PCR, **(A)** 2^{-ΔCT} were plotted for untreated and IL-6 treated and **(B)** fold induction was calculated by dividing 2^{-ΔCT} of IL-6 treated cells by 2^{-ΔCT} of untreated cells, this is equivalent to 2^{-ΔΔCT}. Significance was determined by two-way analysis of variants with Bonferroni posthoc pairwise analyses: ***p<0.0001.

5.3.5 Gene expression changes post-TCR stimulation

Next, genes differentially expressed between the untreated and IL-6 pre-exposed naïve and memory CD4⁺ T-cells at the time-points post TCR stimulation was assessed, including 4 hours, 16 hours and 6 days. When examining differential gene expression at time points following TCR stimulation there were substantially fewer DEGs found after 4 hours and 16 hours and no

DEGs found 6 days following TCR stimulation or 16 hours following TCR stimulation in memory CD4⁺ T-cells, *Table 16*.

4 hours post-TCR stimulation there were 233 DEGs in naïve CD4⁺ T-cells compared to only 10 DEGs in memory CD4⁺ T-cells, *Table 16*. The full list of genes can be seen in *CD-ROM Gene List 1C and 2C*.

The top 10 differentially expressed genes after 72 hours exposure to IL-6 followed by 4 hour TCR stimulation in naïve CD4⁺ T-cells are summarised in *Table 18*. Of these genes, four were chosen for investigation in *Chapter 6* to explore whether these genes were differentially expressed in disease conditions. These genes are termed the “IL-6 post-TCR signature” and their induction was assessed, following 72 hours exposure to IL-6 followed by 4 hour TCR stimulation, by real-time PCR, *Figure 48*. The induction of CST7 and GPT2 was significantly increased by pre-exposure to IL-6 followed by TCR stimulation however there was no induction of GSTO1. In contrast RRAD appeared to show a decrease following IL-6 pre-exposure.

Gene Symbol	Illumina ID	Log Fold Change	P-value
GSTO1*	ILMN_2227573	-1.021102282	2.67199E-06
RRAD*	ILMN_2186137	1.318849708	2.67199E-06
GPT2*	ILMN_1684158	1.432891356	2.67199E-06
ISM1	ILMN_1772869	-0.97596656	6.1381E-06
RASA1	ILMN_1725312	-0.79472334	6.1381E-06
CST7*	ILMN_1679826	1.849028026	6.49498E-06
C1orf22	ILMN_1663068	-0.985021927	1.07571E-05
ARC	ILMN_1711120	1.053244701	2.86219E-05
TMEM194A	ILMN_3228822	-0.751914657	0.000169431
CBS	ILMN_1804735	1.306365759	0.003820756

Table 18. Top 10 differentially expressed genes in naïve CD4⁺ T-cells after 72 hours IL-6 pre-exposure followed by 4 hours TCR stimulation. Genes shown are the top 10 differentially expressed genes with the strongest p values with their respective log fold changes and illumina IDs. Genes highlighted with * represent those genes used for further validation of the IL-6 post-TCR signature in *Chapter 6*.

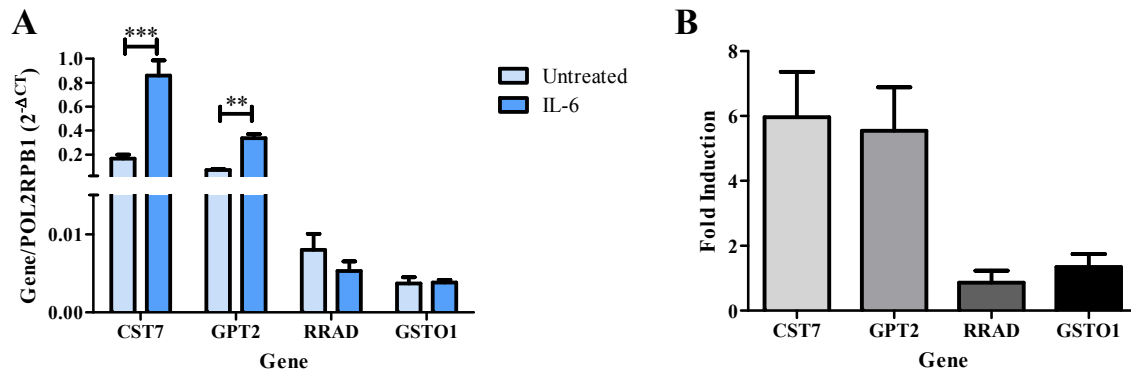


Figure 48. Validation of “IL-6 post-TCR signature” genes in naïve CD4⁺ T-cells following 72 hours IL-6 exposure. Naïve CD4⁺ T-cells from 5 healthy donors were cultured with 0.5ng/ml IL-6 and equimolar sIL-6R for 72 hours, washed and stimulated with 0.2μg/ml anti-CD3 and 1μg/ml anti-CD28 for 4 hours and RNA was extracted. 4 genes shown to be differentially expressed following 72 hours IL-6 exposure and subsequent 4 hour TCR stimulation were assessed by real-time PCR, (A) $2^{-\Delta CT}$ were plotted for untreated and IL-6 treated and (B) fold induction was calculated by dividing $2^{-\Delta CT}$ of IL-6 treated cells by $2^{-\Delta CT}$ of untreated cells, this is equivalent to $2^{-\Delta\Delta CT}$. Significance was determined by two-way analysis of variants with Bonferroni posthoc pairwise analyses: **p<0.001, ***p<0.0001.

The DEGs 4 hours post-TCR stimulation are highly similar between naïve and memory CD4⁺ T-cells with only one of the genes (ANK3) differentially expressed in memory CD4⁺ T-cells not found to be differentially expressed in naïve CD4⁺ T-cells. The full list of these genes can be seen in the CD-ROM in the *Appendix, Gene list 1C and 2C*. Furthermore, only 2 of the 123 DEGs identified as a consequence of IL-6 pre-exposure in naïve CD4⁺ T-cells 4 hours post TCR stimulation were overlapping with the genes previously found by Pratt et. al. to be dysregulated between early RA and non-RA, but not found higher than would be expected by chance. This indicates altered response to TCR stimulation, which is enhanced in naïve CD4⁺ T-cells compared to memory CD4⁺ T-cells. The absence of any DEGs at both 16 hours post-stimulation and 6 days post-stimulation indicates no longer lasting consequences on gene expression as a result of IL-6 pre-exposure.

5.3.6 Gene expression changes over time

A striking observation from the analysis hereto had been the apparent absence of genes differentially expressed in cross sectional analyses at 16 hours or 6 days, *Table 16*. To allay concern that the pairwise analyses undertaken might be insufficiently sensitive to identify all genes whose dynamic profiles differed significantly as a result of IL-6 pre/non-exposure, especially following TCR stimulation, the next analyses carried out was a longitudinal analysis, as shown in *Figure 43*. This longitudinal analysis was carried out with the aim of identifying

those genes differentially induced by TCR stimulus at the 4 hour, 16 hour and 6 day post-TCR time-points relative to the 72 hour pre-TCR time-point. The number of differentially induced genes at each of these time-points relative to the 72 hour pre-TCR time point can be seen in *Table 19*.

Post-TCR time-point	DEG in untreated naïve CD4 ⁺ T-cells	DEG in IL-6 pre-exposed naïve CD4 ⁺ T-cells
4 hours	4318	4457
16 hours	1209	1496
6 days	5641	5325

Table 19. Genes differentially induced in naïve CD4⁺ T-cells post-TCR stimulation relative to 72 hours IL-6 exposure pre-TCR stimulation. Genes were identified which were differentially induced between the 72 hour time point pre-TCR stimulation and all time points post-TCR stimulation in both untreated and IL-6 pre-exposed naïve CD4⁺ T-cells for 3 biological replicates.

Similar to the cross sectional analysis between untreated and IL-6 exposed cells, there were fewer differentially regulated genes at the 16 hour time-point compared to the 4 hour time point however after 6 days stimulation there are much greater numbers of differentially expressed genes. This longitudinal analysis revealed many genes differentially induced at time-points post-TCR stimulation in both untreated naïve CD4⁺ T-cells and a similar number of genes differentially induced in IL-6 pre-exposed naïve CD4⁺ T-cells. This highlights that there are gene expression changes post TCR stimulation as a result of IL-6 pre-exposure, however, a pair-wise test of differential expression is not sensitive enough to detect these changes, as shown in *Table 16*.

Genes whose dynamic profiles differed as a result of TCR stimulation at the 4 hour time point post TCR stimulation compared to the 72 hour pre-TCR time-point are depicted in *Figure 49*. There were 1576 genes up regulated and 1674 genes down regulated irrespective of IL-6 pre-incubation. However, in IL-6 pre-exposed naïve CD4⁺ T-cells there were 646 genes uniquely induced by TCR stimulation and 556 genes uniquely repressed. These lists include some of those found to be differentially expressed in the cross sectional analysis between untreated and IL-6 pre-exposed cells 4 hours post-TCR stimulation, including CST7, ARC, CBS and 12 others. Other notable genes induced at this time point include SOCS3, SBNO2, PRG4, REXO2,

C10orf10 and ARID5B, genes found to be differentially expressed by presence of IL-6 pre-TCR stimulation. Only 3 probes were found to be down regulated in untreated cells but up regulated by IL-6 pre-exposure. Two of these probes corresponded to the master transcription factor of Tfh cells, BCL6. The full list of genes for each time-point can be found in *CD-ROM Gene lists 3A-F*.

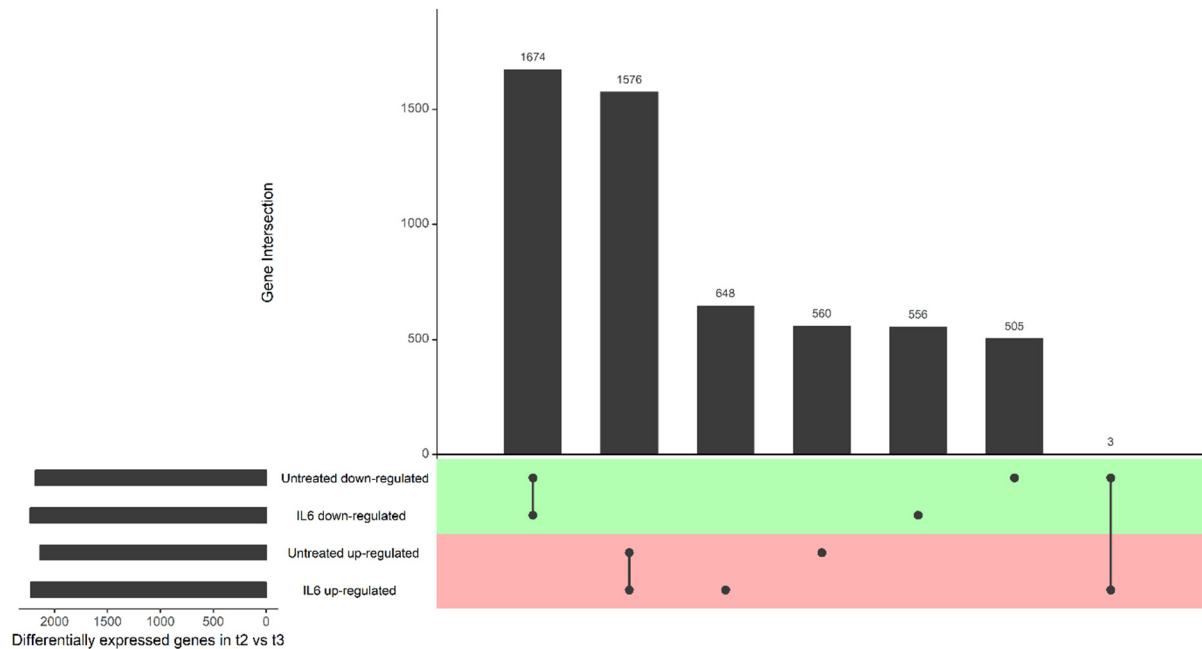


Figure 49. Upset plot of the intersections between gene sets differentially induced 4 hours following TCR stimulation relative to pre-TCR stimulation at 72 hours in naïve CD4⁺ T-cells as a consequence of IL-6 exposure. Differentially expressed genes were determined in both untreated and IL-6 pre-exposed cells between 72 hours pre-TCR stimulation and 4 hours post-TCR stimulation for 3 biological replicates with log₂ FC >1.5 and corrected p<0.05. The bar chart on the left indicates the number of genes up- or down- regulated in untreated or IL-6/sIL-6R pre-exposed naïve CD4⁺ T-cells. The upper bar chart depicts the number of genes in each intersection, as summarised in the lower panel.

Furthermore, there were many genes found to be induced by TCR stimulation in both the untreated and IL-6 pre-exposed naïve and memory CD4⁺ T-cells. These overlapping genes between untreated and IL-6 pre-exposed cells were more abundant at 4 hours and 6 days post-TCR stimulation than genes which are unique to untreated or IL-6 pre-exposed cells. Moreover, despite having fewer genes induced at 4 hours post-TCR stimulation there is a higher number of genes induced by TCR stimulation in both untreated and IL-6 pre-exposed memory CD4⁺ T-cells at 6 days post stimulation.

5.3.7 Pathways differentially induced by IL-6 pre-exposure

Ingenuity Pathway Analysis (IPA) software (Qiagen, Germany) was used to analyse particular DEG lists identified in *Chapter 5.3.3-6*, in order to derive the key biological processes

represented and gain insight into the underlying biology. Pathway analysis identifies related proteins, cellular processes or signalling pathways that are statistically associated with the differences in gene expression between two samples. It requires prior knowledge of biological processes and their interactions and in the cases of GO and KEGG exploit knowledge in public repositories.

The gene lists first subjected to pathway analysis were those identified in *Chapter 5.3.3* in cross sectional differential expression analysis at 6-hour, 72-hour post-IL-6 exposure and 4 hour post-TCR stimulation to reveal the pathways differentially expressed as a consequence of IL-6, these lists can be found in the CD-ROM in *Appendix, Gene Lists 1A-C*. At all times points, STAT-3 was predicted to be a regulator confirming the role of STAT-3 mediated signalling in the IL-6 pre-exposure model.

Biological functions of DEGs obtained with IL-6 pre-exposure compared to non-pre-exposure followed by 4-hour TCR stimulation of naïve CD4⁺ T-cells included cellular movement, cell death and survival, cellular development and cell growth and proliferation, *Figure 50*. Canonical pathways identified at this time point include TCR signalling and many pathways involved in the metabolism of nucleotides suggestive of increased activation status, which correlates with the aberrant effector phenotype described in *Chapter 4*.

There were few/no DEGs found in cross sectional analysis between untreated and IL-6 pre-exposed CD4⁺ T-cells at time-points 16 hours and 6 days post-TCR stimulation, however, a large number of genes were found to be differentially induced by TCR stimulation in longitudinal analysis described in *Chapter 5.3.6*. IPA analysis was then carried out on genes found to be differentially induced at the 4 hour, 16 hour and 6 day post-TCR time-points relative to the 72hr pre-TCR time-point for both untreated and IL-6 pre-exposed naïve CD4⁺ T-cells to identify pathways differentially induced by IL-6 pre-exposure at later time-points post TCR stimulation.

The IPA analysis on genes differentially induced uniquely with IL-6 pre-exposure at 4 hours post-TCR compared to 72 hours pre-TCR revealed decreased lymphocyte apoptosis and decreased lymphocyte migration with p-value of 1.23E-32 and z-score of -2.676 or p value 8.04E-16 and z-score -2.345 respectively. In contrast, these pathways were not identified in genes uniquely induced in untreated naïve CD4⁺ T-cells with decreased quantities of T-helper cells identified with p-value 1.58E-05 and z-score of -2.507.

IPA analysis on genes differentially induced uniquely in IL-6 pre-exposed naïve CD4⁺ T-cells at 16 hours post TCR stimulation compared to 72 hours pre-TCR stimulation revealed increased

cell adhesion, cell movement and increased quantity of leukocytes with p-values of 5.65E-05, 1.56E-04 and 1.38E-05 respectively and z-scores of 2.047, 1.567 and 2.182 respectively. In addition to these differentially induced biological functions predicted upstream regulators of this pathway were suggested to be TCR stimulation and IL-6 stimulation and the STAT-3 pathway was also significantly upregulated at this time-point post-TCR stimulation, providing reassurance that the effects seen are a result of IL-6 mediated STAT-3 signalling.

This is in contrast to genes differentially induced uniquely in non-pre-exposed naïve CD4⁺ T-cells at 16 hours post-TCR stimulation compared to 72 hours pre-TCR stimulation which were included decreased cell viability and decreased T-cell proliferation.

Finally, following 6-day post-TCR stimulation genes which were uniquely differentially induced at this time-point relative to the 72-hour time-point as a result of IL-6 pre-exposure were analysed. IPA analysis found these genes to be involved in decreased immune response of cells and increased cell death of connective tissue cells with p-values of 6.28E-03 and 4.76E-04 respectively and z-scores of -2.670 and 1.650 respectively, suggesting potential pathogenic effects of IL-6 pre-exposure. On the other hand, pathways differentially induced uniquely in non-pre-exposed naïve CD4⁺ T-cells 6 days post-TCR stimulation involved decreased cell survival with a p-value of 4.21E-04 and a z-score of -2.008. In addition, to increased cell death, joint inflammation was also described as a pathway increased in non-pre-exposed naïve CD4⁺ T-cells 6 days post-TCR stimulation with a p-value of 2.12E-06 and z-score of 1.901. The increased joint inflammation pathway contained 41 genes, of which only 4 genes were found to result in the activation of this pathway, whereas the effect could not be predicted for the remaining 37 genes.

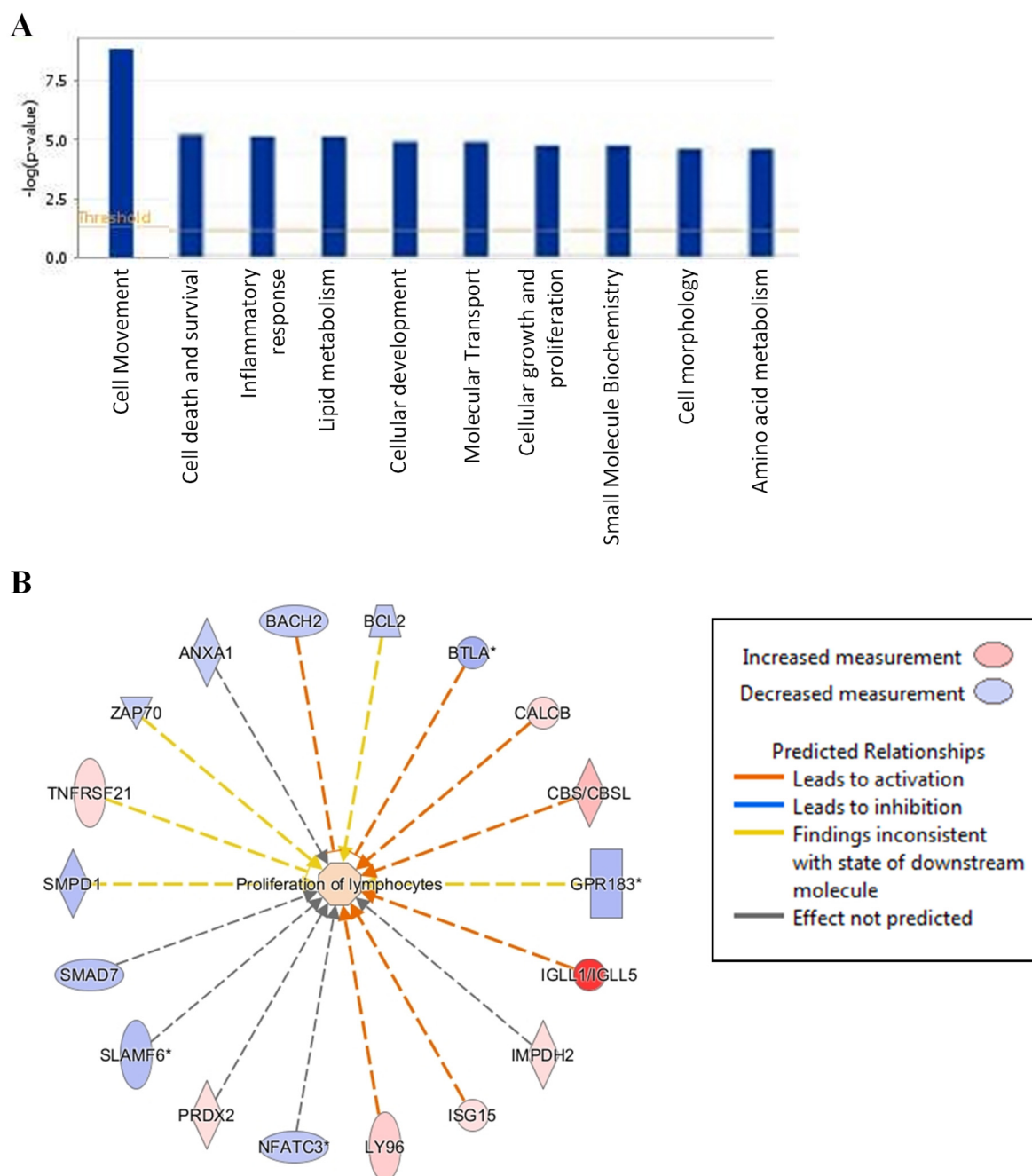


Figure 50. Pathway analysis of differentially expressed genes in naïve CD4⁺ T-cells as a result of IL-6 pre-exposure and 4 hours TCR stimulation. DEG lists from cross sectional differential expression analysis of IL-6 pre-exposed naïve CD4⁺ T-cells at 4 hours post-TCR stimulation for 3 biological replicates were subject to IPA analysis. This analysis revealed numerous genes involved in pathways relating to cell proliferation, movement and survival (A) There were 18 genes found to be differentially up-regulated (pink) or down-regulated (blue) between untreated and IL-6 pre-exposed naïve CD4⁺ T-cells which were associated with lymphocyte proliferation (B). The consequence of the expression of the molecule on the proliferation pathway is indicated by the colour of the line with orange being activation of proliferation, yellow being inconsistent with the state of the pathway and grey indicating that the effect on the pathway could not be predicted.

5.4 Discussion

The aims of this Chapter were to investigate how the effector phenotype of naïve and memory CD4⁺ T-cells following IL-6 pre-exposure was regulated by differential gene expression in these cells. A microarray of RNA extracted at several time points post-IL-6 exposure and post-TCR stimulation was undertaken which revealed a number of genes differentially expressed as a result of IL-6 pre-exposure. A number of the genes differentially expressed in the presence of IL-6 in both naïve and memory CD4⁺ T-cells overlap with genes previously found by Pratt et al. to discriminate early RA from disease controls. IL-6 pre-exposure also has consequences on gene expression following 4-hour TCR stimulation, however, no genes can be found in cross-sectional analyses at 16 hours or 6 days post-TCR stimulation. In a longitudinal analysis of the later time-points post-TCR stimulation many genes were found to be differentially induced uniquely by IL-6 relative to the 72-hour pre-TCR stimulation time-point. Many of the genes found to be differentially induced by IL-6 pre-exposure are those involved in proliferation, cell development and survival, which reinforce the consequences on phenotype seen in *Chapter 4*. Interestingly, naïve CD4⁺ T-cells have a much higher number of differentially expressed genes following IL-6 pre-exposure compared to memory CD4⁺ T-cells. This is consistent with data suggesting naïve CD4⁺ T-cells are more sensitive to IL-6 pre-exposure compared to memory CD4⁺ T-cells, *Figure 8, Chapter 1.3.12*. However, although memory CD4⁺ T-cells have a lower number of differentially expressed genes following IL-6 pre-exposure a large proportion of these overlap with previously identified RA signature genes indicating that the influence of IL-6 upon both circulating subsets probably contributes to the previously described discriminatory gene signature.

5.4.1 Design of dynamic CD4⁺ T-cell transcriptome experiment

Profiling the transcriptome represents a valuable means of understanding biological processes. Many high throughput sequencing technologies have been developed to profile cell gene expression including microarray and RNA sequencing. The microarray is a popular and relatively low cost technique providing reliable data on relative gene expression in a particular cell population. One disadvantage of this method is its dependence on probes designed using prior knowledge of the target organism's genome; low abundance transcripts or splice variants are not always captured. In addition, issues regarding probe sensitivity and specificity have arisen, with a number of probes able to cross-hybridise with unintended targets across the genome, these probes were removed from the analysis in this Chapter.

In contrast, RNA-seq is where transcripts are directly sequenced to quantify absolute and relative gene expression. This technique is fast with high sensitivity and does not require prior

knowledge of transcripts. RNA-seq is now the favoured method of gene expression analysis. The work in this thesis was designed and undertaken during the time of transition from microarray to RNA-seq. The considerations of cost and analytical challenge, historically causing researchers to favour microarray have largely been overcome making it conceivable that RNA-seq may have been favoured if the study were designed again. Due to the fact that splice variant information was not required and the abundant in-house understanding of the Illumina microarray technology the decision was made to use the microarray technique for genetic analysis.

Although there are clear differences in RNA seq and microarray technology with RNA seq providing a more dynamic range for DEG identification (509) microarrays are still robust and reliable tools for differential gene expression analysis with studies describing strong overlap of DEGs between the two techniques (510). Transcript abundance between the different gene expression technologies are highly correlated and 80% of genes differentially expressed by microarray were detected by sequencing (511). Differences occur when high intensity probes are identified with only small sequence counts, this may be due to the background hybridisation of probes which was accounted for in our analysis, with others stating that concordance depends on treatment and transcript abundance (512).

Misinterpretations of data can occur due to the presence of contaminating cell populations. In cell isolations using positive selection monocytes are often contaminating cell populations in CD4⁺ T-cell isolations due to their expression of CD4 which has the potential to influence DEG lists (461). Although positive selection was reviewed to have no significant influence on gene expression (513) our study favoured negative selection of naïve CD4⁺ T-cells which resulted in over 90% purity with few contaminating monocytes or B-cells. Despite highly pure naïve CD4⁺ T-cells the memory CD4⁺ T-cell populations have lower purity with contaminating populations, mainly naïve CD4⁺ T-cells, due to this the data should be interpreted with caution.

Another issue which may confound microarray analysis is both the sample processing and the delays from blood draw to cell separation. This variable affects many cell types and has been shown to decrease expression of certain genes even between 1-3 hours, however, lymphocyte transcriptomes are stable for longer in contrast to monocytes (514, 515). Therefore, all isolations were carried out immediately following blood draw with as much efficiency as possible.

In summary, although newer sequencing technologies now exist the careful experimental design and good quality control data obtained using the microarray approach provide

confidence in the integrity of the data. This has provided an extensive data set enriched for genes differentially expressed as a result of IL-6 pre-exposure.

5.4.2 Gene expression analysis

In the first stages of quality control, *Chapter 5.3.2*, it was shown that donor 4 appeared not to respond in terms of gene expression in the same way as donors 1-3 to TCR stimulation. This donor did appear to respond to stimulation in the CFSE proliferation assay with a fold change of three, due to this it is possible that there was an experimental error that occurred during experimental set up that resulted in the cells from which RNA was taken were not stimulated.

5.4.2.1 Gene expression changes with IL-6 exposure

A number of genes found to be differentially expressed following exposure to IL-6 at 6 hours or 72 hours, were those previously described as being up- or down- regulated by IL-6 such as ARID5B, ID2, BCL3, BCL6, STAT3, STAT4 and the IL-21R (385). The full list of DEGs can be found in Genes lists 1A and B and 2A and B, *Appendix*. In addition to the induction of ARID5B, in both naïve and memory CD4⁺ T-cells, an important paralog of this gene ARID5A was differentially expressed following IL-6 exposure specifically in naïve CD4⁺ T-cells. This gene has recently been shown to regulate naïve CD4⁺ T-cell fate in an IL-6 dependent manner by the selective stabilisation of STAT-3 resulting in the differentiation of cells into inflammatory cells, particularly Th17 cells (345). This indicates the role of IL-6 pre-exposure in development of a more pathogenic pro-inflammatory effector cell.

A notable finding was the striking induction of BCL6 both with IL-6 exposure prior to TCR stimulation and its upregulation following 4 hours TCR stimulation compared to its downregulation in untreated cells. This confirms the importance of IL-6 as a key driver of Tfh cells, however no difference was seen in Tfh differentiation with IL-6 pre-exposure, *Chapter 4 (162)*.

Many genes previously described as being induced by IL-6 were not observed as differentially expressed in this study including IL-21, CCR5, ROR- γ/α , CD4, STAT-4 and FasL. This may be due to the difference in experimental set up, which in this study involves the prolonged exposure of naïve CD4⁺ T-cells to IL-6 for 72 hours whereas in other studies may have different lengths of IL-6 stimulation or involve the presence of IL-6 at the time of stimulation. Another factor is the concentration of IL-6 used which in this study was 0.5ng/ml as this was deemed to be more physiological compared to others which use supra-physiological concentrations of 100ng/ml. The simultaneous stimulation of cells with both IL-6 and via their TCR may be needed to result in the differential expression of these genes and the prolonged exposure to IL-

6 prior to stimulation may mean this is not seen. One potential limitation of this system is that IL-6 is only added at the beginning of culture and not refreshed therefore effects may be lost after 72 hours.

In addition to the expression of previously identified IL-6 regulated genes a number of STAT-3 regulated genes were identified which overlap with genes previously found by Pratt et al. in total CD4⁺ T-cells to distinguish untreated early RA patients from disease controls (431). These include BCL3, PIM1, SBNO2 and MUC1 and the presence of these genes was significantly higher than would be expected by chance providing validation that the gene signature seen in these patients may be driven by IL-6 (431), highlighting the importance of this cytokine in disease the indicating the relevance of IL-6 pre-exposure in this disease setting.

5.4.2.2 Gene expression changes post TCR stimulation

With the exception of donor 4, which was excluded from differential gene expression analysis, when principle component analysis was carried out upon remaining samples TCR-mediated activation was a strong principle component identifier. This is likely to be due to extensive transcriptional changes documented upon activation. Wang et al. identified 1463 genes with significant expression changes following activation with anti-CD3 and anti-CD28 beads over 0-72 hours in CD4⁺ T-cells and 3793 genes in CD3⁺ T-cells (516). Among these genes were immune response genes, cytokines and receptors. Many of these genes were also found to be differentially expressed in response to TCR stimulation here including CD69, CD83, BCL6 in untreated naïve CD4⁺ T-cells at 4 hours and ITGAL, ICOS and CTLA4 in both untreated and IL-6 pre-exposed naïve CD4⁺ T-cells at 4 hours. This provides reassurance that cells have responded as expected to TCR stimulation.

In particular, Hess et al., using a microarray containing 5016 cDNA clones, reported many genes induced during early, intermediate and late phases of anti-CD3 and anti-CD28 activation, 15, 25 and 156 genes respectively making a total of 196 DEGs with more genes differentially induced as time from activation increased (517). Data presented in this Chapter is similar with the number of differentially induced genes is much higher after 6 days stimulation compared to 16 hours post stimulation. Of the 196 genes, 10 genes were found in this Chapter to be induced by TCR stimulation, including JAK1, IL10RA and PSMA3. Diehn et al. also profiled gene expression changes following anti-CD3 and anti-CD28 stimulation at 7 time points between 0-48 hours finding 2926 genes differentially expressed by 3-fold (518). Many of the genes induced include cytokines, cell adhesion molecules, effector molecules, cell cycle and proliferation molecules as well as metabolic elements such as glycolysis.

Data presented here show lower numbers of DEGs, in the range of 20-421 DEGs, in response to TCR mediated stimulation. A reason for this difference may be in part down to the proportion of responding cells in the system. Following TCR stimulation, as indicated by the proliferation graphs, *Chapter 5.3.1*, only up to 5% of untreated cells and 10-20% of IL-6 pre-exposed cells are dividing in the final population. As a result, the non-activated cells will be causing a dilution in the signal from the responsive cells, which may lower the differences in fold changes between untreated and IL-6 pre-exposed cells. The proportion of responding cells investigated by Diehn et al. was not included in analysis, however, is potentially higher than the number seen in this study due to the stronger stimulus used. Diehn et al. stimulated cells with anti-CD3 and anti-CD28 coated beads at a ratio of 1:1 which is a much stronger stimulus than the relatively low stimulation concentration of anti-CD3 and anti-CD28 used in this Chapter. Another reason for this difference may be the type of culture medium used, the in vitro model presented in this thesis applies the use of serum free medium which may result in differences in gene expression post-TCR stimulation.

The lower number of DEGs identified is supported in the literature as Allison et al. showed that the affinity and dose of the TCR stimulation will govern the magnitude of response, with stronger TCR signals resulting in higher amounts of phosphorylated ERK, enhancer acetylation and activation markers in CD4⁺ T-cells (519). In the experiment presented in this Chapter, however, the level of TCR stimulation was optimised as being 0.2µg/ml anti-CD3 and 1µg/ml anti-CD28, significantly lower than optimised in the in vitro model, Chapter 3.4.4. This was in order to optimise the model for use of whole blood drawn in citrate anti-coagulant, as opposed to LRS cones used in the in vitro model. As a result, the relatively low stimulus may have resulted in a lower magnitude response of the naïve and memory CD4⁺ T-cells. It is possible that with a higher concentration of stimulus the greater response would have allowed the identification of more DEGs mediated by IL-6 pre-exposure, particularly at later time-points post-TCR stimulation. At day 6 where it was shown in *Chapter 4* that cells have increased proliferative capacity and differential activation marker and transcription profiles cells did not have any differentially expressed genes with IL-6 pre-exposure using the cross sectional paired t-test to identify differentially expressed genes, *Chapter 5.3.3*. These differences can be detected by differential gene induction in untreated and IL-6 pre-exposed cells, *Chapter 5.3.6*, suggesting that the paired differential expression test is not sensitive enough to detect significant changes in cross sectional analysis, potentially due to the high level of multiple test correction. This indicates that although there are no significant differentially expressed genes

at later time points post-TCR gene expression changes are still occurring which impact on the effector function of the CD4⁺ T-cell.

5.4.2.3 Pathways highlighted by IL-6 pre-exposure

The pathways identified in this Chapter as being significantly up or down regulated in naïve CD4⁺ T-cells pre-exposed to IL-6 involve increased survival, maturation, migration and proliferation of lymphocytes, which reflects the aberrant phenotype of IL-6 pre-exposed naïve CD4⁺ T-cells seen in *Chapter 4*. Further pathways identified at 6 days post-TCR stimulation with IL-6 pre-exposure involve the decreased immune response of cells and increased cell death of connective tissue cells. This fits in with the pathology of rheumatoid arthritis whose CD4⁺ T-cells have been well described in the literature as being phenotypically exhausted and less responsive to stimulation (111, 520). In contrast, the pathways found to be significantly up or down regulated in untreated naïve CD4⁺ T-cells involved decreased cell viability and decreased T-cell proliferation reflecting the phenotype of untreated naïve CD4⁺ T-cells, *Chapter 4*. Pathways involving joint inflammation were found to be upregulated in untreated naïve CD4⁺ T-cells 6 days post-TCR stimulation which does not fit in with IL-6 pre-exposure causing more pathogenic phenotype, however, only 4 of the 41 genes were shown to confidently up-regulate this pathway with all other genes having no predicted effect. Therefore, this pathway cannot be confidently described as upregulated in untreated naïve CD4⁺ T-cells.

Finally, there were more DEGs identified in naïve compared to memory CD4⁺ T-cells as a consequence of IL-6 pre-exposure, *Table 16*. The greater transcriptional response to IL-6 seen in naïve CD4⁺ T-cells is consistent with the notion that these cells have increased sensitivity to circulating IL-6 which may relate to the expression of gp130 signalling receptor as previously described in *Chapter 3.3.1*.

It has been shown that inflammatory cytokines, such as TNF, can alter the levels of DNA methyltransferases causing altered methylation of specific promoters, which underlie cellular dysregulation in cardiomyocytes (521). A similar mechanism may also be possible with IL-6 with studies showing that IL-6 has the ability to increase DNA methyltransferase expression and activity (522, 523). This alteration of DNA methyltransferases may occur in CD4⁺ T-cells mediating the methylation of promoter regions of cell cycle regulatory genes to result in enhanced proliferative capacity.

5.4.3 Summary

In summary, here it is shown that IL-6 pre-exposure causes differential gene expression in CD4⁺ T-cells that is most abundant in naïve cells, indicating increased transcriptional sensitivity to

the cytokine in this sub-population. The differentially expressed genes following IL-6 pre-exposure strongly reflect those genes found to be differentially expressed in early RA patients, providing independent validation of the relevance of IL-6 signalling in early RA pathogenesis. There was also a distinctive set of genes differentially expressed 4 hours post-TCR stimulation in cells pre-exposed to IL-6 which were involved in cell proliferation and growth, suggesting IL-6 pre-exposure programmes CD4⁺ T-cells with an enhanced proliferative capacity following activation.

Chapter 6. Effects of IL-6 pre-exposure in relation to rheumatoid arthritis

6.1 Introduction

Work outline in previous chapters has demonstrated that chronic pre-exposure of healthy control naïve and memory CD4⁺ T-cells to physiological concentrations of IL-6 and equimolar sIL-6R has consequences on effector cell phenotype and gene expression following TCR stimulation. Specifically IL-6 pre-exposure results in increased proliferative capacity, activation marker and transcription factor expression of both naïve and memory CD4⁺ T-cells. IL-6 pre-exposure of memory CD4⁺ T-cells results in altered cytokine production whereas pre-exposure of naïve CD4⁺ T-cells has no influence on cytokine production following TCR stimulation. When differentiated towards T-helper cell subsets the pre-exposure of naïve CD4⁺ T-cells to IL-6 results in increased IFN- γ production of Th1 cells whereas the pre-exposure of memory CD4⁺ T-cells results in the inhibition of IL-17 production from Th17 cells. These effects of IL-6 pre-exposure on effector phenotype have been shown to be mediated by a subset of genes both pre- and post- TCR-stimulation involved in cell development, survival and proliferation and many of the genes found upon IL-6 exposure overlap with genes previously described as dysregulated in early RA.

These findings were of interest, particularly when considered alongside previously published observations regarding the CD4⁺ T-cell phenotype in early and established RA. It has been reported that in RA inflammation drives naïve CD4⁺ T-cell proliferation, evidenced by the reduced levels of TREC, promoting the differentiation of atypical progeny, which are hypo-responsive to ex vivo stimulation (108). Furthermore, there is strong evidence to suggest the acceleration of immunosenescence in RA (524). Naïve CD4⁺ T-cells have restricted diversity and clonal burst and the replicative stress experienced by these cells may lead to pathogenic senescent subsets which have reduced thresholds of activation and are supported by the optimal conditions of the synovial micro-environment. Evidence for the immunosenescence of effector subsets of CD4⁺ T-cells is shown by reduced cell cycle duration, reduced numbers of cells dividing and fewer number of divisions per dividing cells of ex vivo stimulated CD4⁺ T-cells from undifferentiated arthritis patients which progress to RA compared to those patients which progress to non-RA disease groups (114). A reason for this apparent immunosenescence may be the cells have already encountered IL-6 in vivo in the periphery and subsequent TCR stimulation secondary lymphoid organs so that when stimulated ex vivo they have decreased responsiveness. The IL-6 pre-exposure in vitro model is based on primary stimulation of naïve

CD4⁺ T-cells following IL-6 exposure, the restimulation of cells may show reduced ability to activate and proliferate compared to cells that have not been exposed to IL-6.

Based on these findings there may be reason to believe that the phenotype of naïve CD4⁺ T-cells stimulated via the TCR following IL-6 pre-exposure, mimicking what is seen in early RA, may recapitulate the altered proliferative phenotype described in RA. Defining the relevance of these findings to the pathogenesis of early RA remains an important question to address.

6.2 Aims and objectives

In light of the above, the over-arching aim of the work carried out in this Chapter is to explore the extent to which the consequences of IL-6 pre-exposure determined by the in vitro model are recapitulated amongst early RA patients in terms of IL-6 receptor expression, proliferative capacity of CD4⁺ T-cells and dynamic gene expression following TCR stimulation.

The observations outlined in this thesis raise the hypothesis that increased levels of circulating IL-6 in RA patients will result in aberrant effector phenotype and gene expression of CD4⁺ T-cells of early RA patients. Due to this, the work carried out in this chapter aims to investigate how these findings are correlated with the situation in naïve and memory CD4⁺ T-cells obtained from early rheumatoid arthritis patients. To do this CD4⁺ T-cells of untreated early RA patients were assessed in terms of cell surface marker expression, proliferation and gene expression. Findings were compared to those in cells from healthy controls as well as from patients with clinically suspect arthralgia (CSA) – defined in this study as those positive for ACPAs who have yet to manifest clinical signs of RA.

Specifically, the objectives were:

- To measure the expression of gp130 and IL-6R on the surface of CD4⁺ T-cell subsets in early arthritis patients and healthy controls and identify any correlation with disease phenotype or clinical characteristics.
- To compare the proliferative response of CD4⁺ T-cells from early untreated RA patients, CSA patients and healthy controls following TCR stimulation. The extent to which observations in CD4⁺ T-cells of patients mirror those in healthy cells pre-exposed to IL-6 (*Chapter 4*) will be assessed.
- Similarly, to compare the expression pattern of a specific set of IL-6 pre/post-TCR signature genes identified in *Chapter 5* (found to be differentially induced by IL-6 pre- and post-TCR stimulation) between CD4⁺ T-cells from healthy donors, CSA and early-untreated RA patients.

6.3 Results

6.3.1 Expression of gp130 and IL-6R on CD4⁺ T-cell subsets of early arthritis clinic attendees

In *Chapter 3.3.1-2*, gp130 and IL-6R expression levels on isolated naïve CD4⁺ T-cells or CD4⁺ T-cells of patients from the NEAC were assessed. This revealed maximal expression of gp130 on true naïve CD4⁺ T-cells compared to central memory and even further reduced levels on effector memory CD4⁺ T-cells, *Figure 12*. Examination of the IL-6R revealed the lowest expression on true naïve CD4⁺ T-cells and highest expression on central memory CD4⁺ T-cells with moderate levels on effector memory CD4⁺ T-cells, *Figure 12B*.

Here, *as in Chapter 3.3.1*, expression of gp130 and IL-6R were assessed on true naïve CD4⁺ T-cells. It was investigated whether any clinical parameters could account for the expression of gp130 or IL-6R. The first analysis to be undertaken was the expression of gp130 and IL-6R stratified by disease diagnosis, *Figure 51*. Gating strategy can be seen in *Figure 8, Appendix* and representative plots *Figure 12, Chapter 3*. The level of expression of both gp130 and IL-6R did not correlate with disease diagnosis, with no difference seen in expression of either marker in EAC attendees compared to healthy controls. Next, it was assessed whether gp130 and IL-6R expression were correlated with C-reactive protein (CRP), a measure of inflammation, or CD25 expression, a marker of cell activation. This shows that there is no significant correlation of gp130 or IL-6R expression levels with CRP, *Figure 52*. Furthermore, there was no correlation of gp130 or IL-6R expression with CD25, *Figure 53*. This is in contrast to previous work showing CD25 negative cells have higher expression of both gp130 and IL-6R (455).

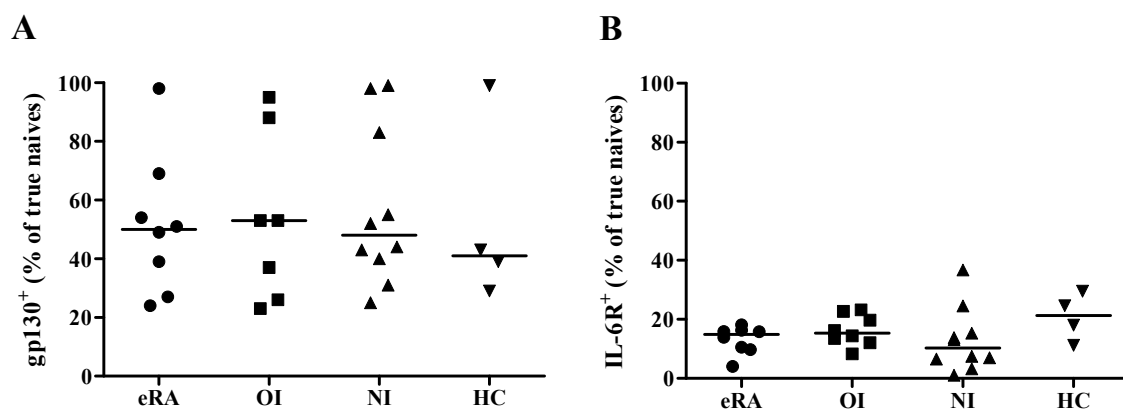


Figure 51. Expression of gp130 and IL-6R on true naïve CD4⁺ T-cells of early arthritis patients according to disease diagnosis. Whole blood of 8 early RA (eRA), 8 other inflammatory (OI), 10 non-inflammatory (NI) and 4 healthy controls (HC) was stained for flow cytometry with a panel of antibodies to detect cell surface expression of gp130 (A) and IL-6R (B). CD4⁺ T-cells were identified as CD3⁺CD4⁺ and from this population true naïve cells were identified as CD45RA⁺CD62L⁺. Values were determined using percentage positive dot plots plotted with median values indicated by the horizontal line.

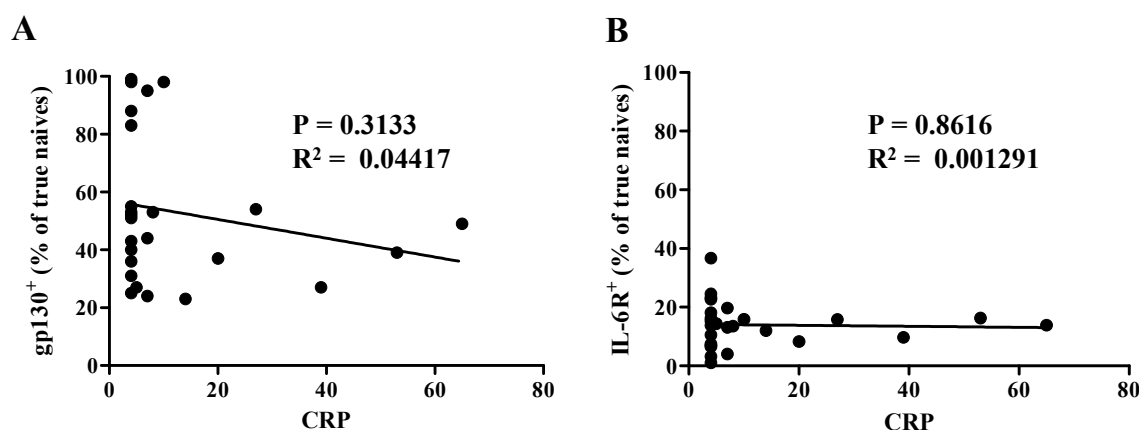


Figure 52. Expression of gp130 and IL-6R on true naïve CD4⁺ T-cells of early arthritis patients plotted against CRP levels. Whole blood of 8 early RA (eRA), 8 other inflammatory (OI), 10 non-inflammatory (NI) was stained for flow cytometry with a panel of antibodies to detect cell surface expression of gp130 (A) and IL-6R (B). CD4⁺ T-cells were identified as CD3⁺CD4⁺ and from this population true naïve cells were identified as CD45RA⁺CD62L⁺. Values were determined using percentage positive dot plots and plotted with a line of linear regression added, gp130: r^2 0.04417 and IL-6R: r^2 0.001291.

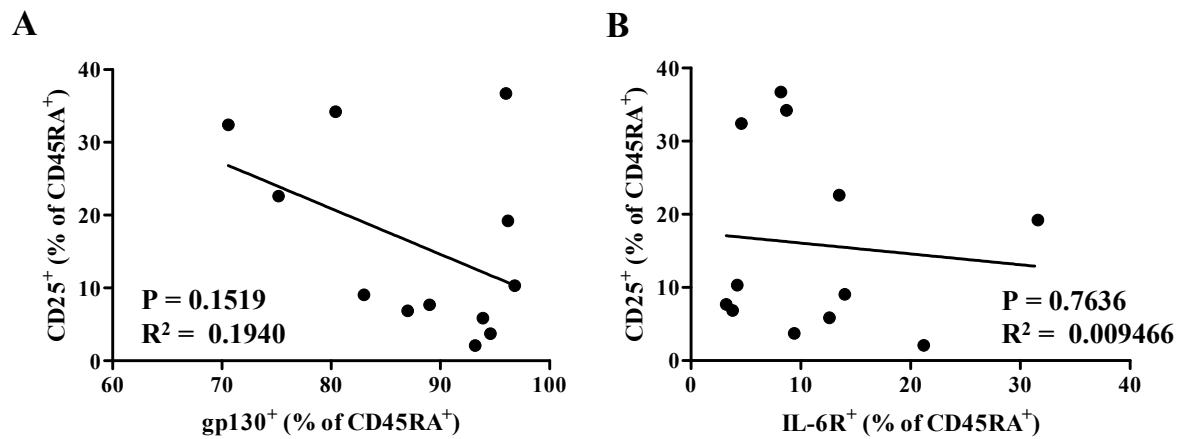


Figure 53. Expression of gp130 and IL-6R on naïve CD4⁺ T-cells of healthy controls plotted against expression of CD25. Naïve CD4⁺ T-cells isolated from 12 healthy controls were stained for flow cytometry with a panel of antibodies to detect cell surface expression of CD25, gp130 (A) and IL-6R (B). CD4⁺ T-cells were identified as CD3⁺CD4⁺ and from this population naïve cells were identified as CD45RA⁺. Values were determined using percentage positive dot plots and plotted with a line of linear regression added, gp130: r^2 0.1940 and IL-6R: r^2 0.009466.

In summary, the level of expression of the IL-6 signalling receptors did not correlate with disease diagnosis or with level of CRP or CD25 expression.

6.3.2 Response of CD4⁺ T-cells from early arthritis clinic attendees to TCR stimulation

Pre-exposure of naïve and memory CD4⁺ T-cells to IL-6 results in increased levels of proliferation following TCR stimulation, *Chapter 4.3.2*. As early rheumatoid arthritis patients have high levels of IL-6 in serum it was hypothesised that levels of proliferation of CD4⁺ T-cells from early RA patients would be similar to levels seen in IL-6 pre-exposed healthy control cells and also be greater than that from healthy controls. In a deviation from previous Chapters proliferation of *total* (as opposed to naïve) CD4⁺ T-cells from early RA patients were assessed due to the relatively limited amount of blood able to be obtained from these patients which would not allow for isolation of naïve CD4⁺ T-cells. Total CD4⁺ T-cells were isolated CFSE labelled and stimulated immediately ex vivo with 0.5µg/ml anti-CD3 and 1µg/ml anti-CD28 for 6 days. After 6 days, proliferation was assessed by flow cytometry.

CD4⁺ T-cells from early RA patients proliferate to a similar extent to healthy controls, with no difference in the number of cells dividing or the number of divisions, *Figure 54*. This suggests that in early RA T-cells have a similar ability to proliferate in response to TCR stimulation as healthy controls. The level of IL-6 in the serum of the early RA patients and healthy controls whose CD4⁺ T-cells were used for proliferation analysis was also assessed, *Figure 54C*. Serum

levels of IL-6 range from 0-70pg/ml, lower than the concentrations used in the in vitro model of IL-6. IL-6 levels also shows dichotomy in with 3 of the early RA patients having higher levels of IL-6 compared to 3 early RA patients and healthy controls. Despite levels of IL-6 being increased in a number of early RA patients compared to healthy controls, there was no correlation between the level of circulating IL-6 and the proliferation of CD4⁺ T-cells.

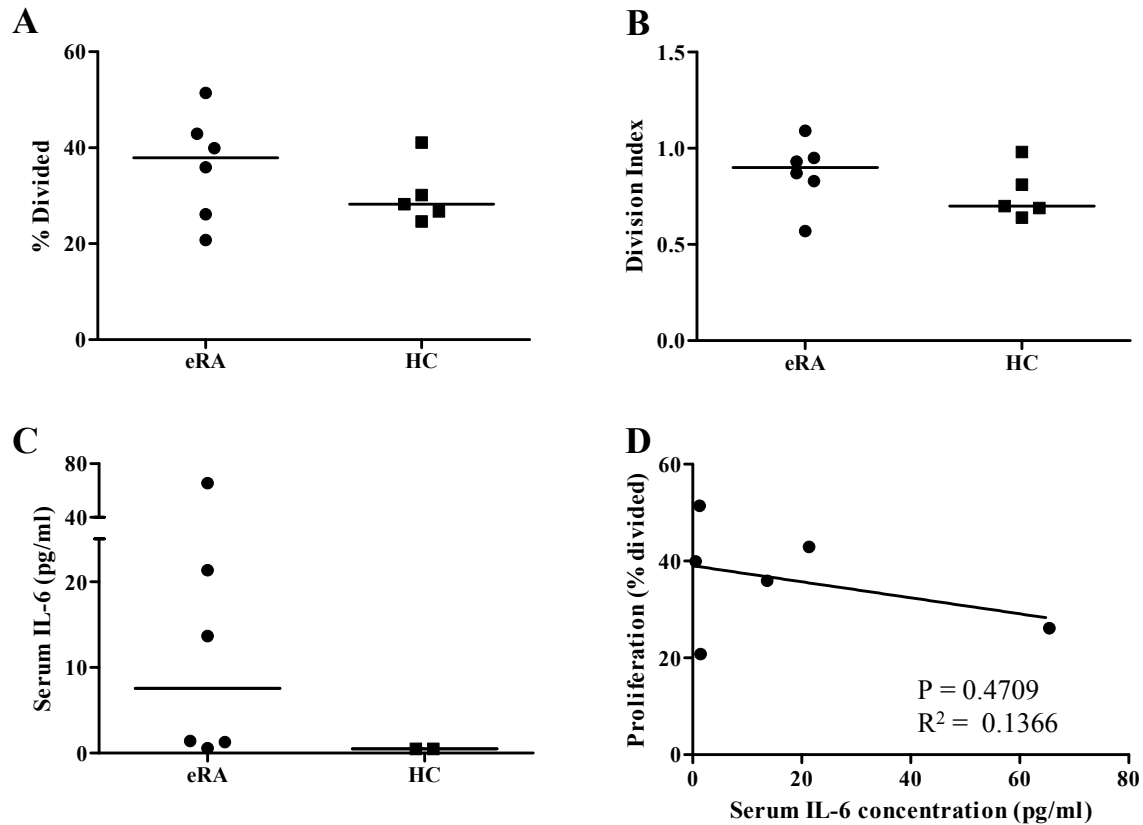


Figure 54. Proliferation of CD4⁺ T-cells of early rheumatoid arthritis patients compared to healthy controls. Total CD4⁺ T-cells isolated from 6 early RA (eRA) patients and 5 healthy controls (HC) were CFSE labelled and stimulated for 6 days with 0.5µg/ml anti-CD3 and 1µg/ml anti-CD28. After 6 days cells were harvested and ran on the flow cytometer. Proliferation was calculated using FlowJo with percentage divided indicating the precursor frequency of dividing cells (**A**), division index showing the average number of divisions (**B**). Serum IL-6 from 6 early RA patients and 2 HC was measured by MSD (**C**) and levels of IL-6 were plotted against level of proliferation (**D**). Significance was determined by Mann Whitney U test. A line of linear regression added r^2 0.1917.

Additionally, CSA patients were recruited to assess levels of proliferation at the early stages of disease pathogenesis. In this instance naïve CD4⁺ T-cells were isolated from these patients as more blood was able to be obtained. These cells were then CFSE labelled and cultured for 3 days in the presence or absence of 0.5ng/ml IL-6 followed by 6 day stimulation with 0.2µg/ml anti-CD3 and 1µg/ml anti-CD28. Here, it can be seen that CSA patients have differing responses to stimulation with some having high levels of proliferation and some having only very low levels of proliferation, *Figure 55A-B*. Levels of proliferation do appear to be marginally higher than that of healthy controls stimulated with the same concentration of anti-CD3 and anti-CD28 seen in *Chapter 5.3.1*. The level of IL-6 in the serum of CSA patients whose naïve CD4⁺ T-cells were used for proliferation analysis was also assessed, however as there were only 3 patients this showed no correlation between serum IL-6 levels and CD4⁺ T-cell proliferation, *Figure 55C*.

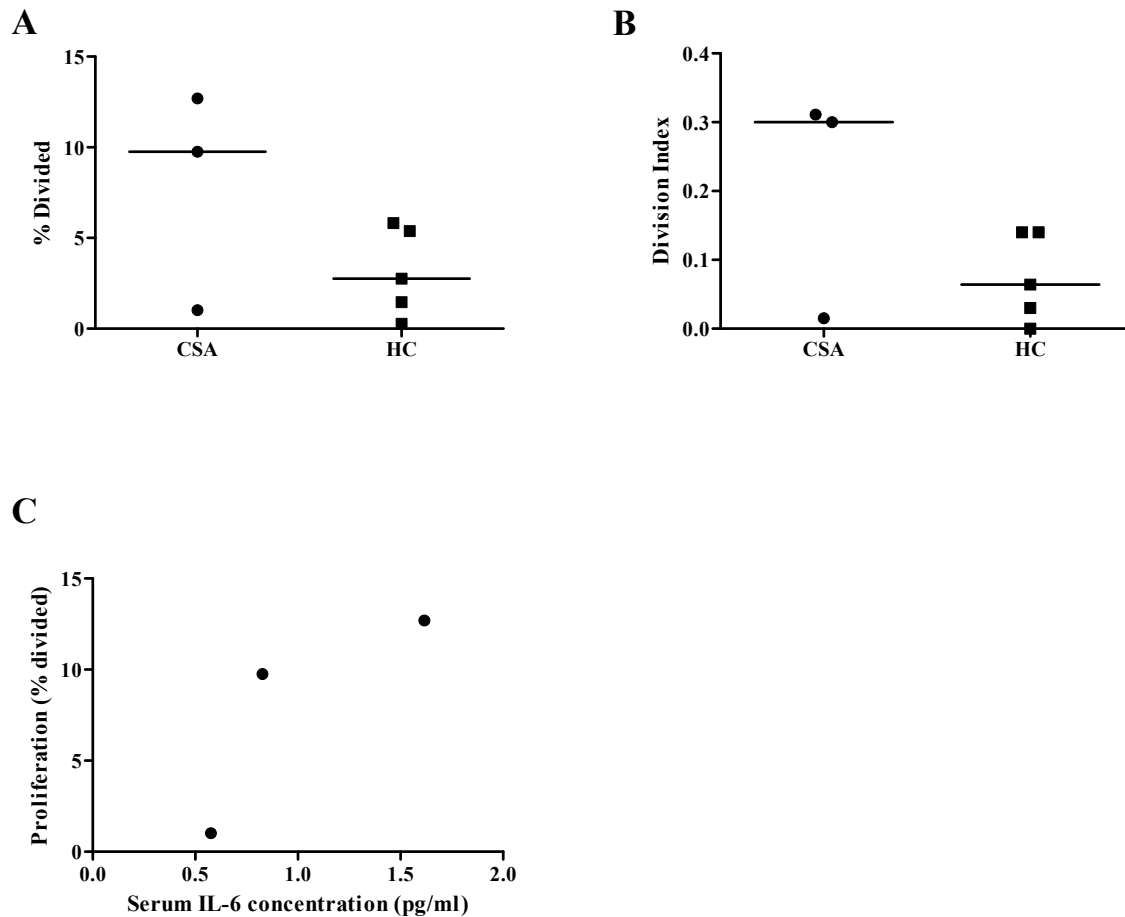


Figure 55. Proliferation of naïve CD4⁺ T-cells of CSA patients compared to healthy controls. Naïve CD4⁺ T-cells isolated from 3 CSA patients and 5 healthy controls (HC) were CFSE labelled cultured for 3 days in absence or presence of 0.5ng/ml IL-6 with equimolar sIL-6R. After 72 hours cells were washed and stimulated for 6 days with 0.2µg/ml anti-CD3 and 1µg/ml anti-CD28. After 6 days cells were harvested and ran on the flow cytometer. Proliferation was calculated using FlowJo with percentage divided indicating the precursor frequency of dividing cells (**A**), division index showing the average number of divisions (**B**). Serum IL-6 from each CSA patient was measured by MSD and correlated with level of proliferation. Significance was determined by Mann Whitney U test.

Finally, in order to assess the ability of patient CD4⁺ T-cells to respond to further IL-6 exposure naïve CD4⁺ T-cells from CSA patients were pre-exposed to 0.5ng/ml IL-6 and equimolar sIL-6R for 3 days, as in the in vitro model, prior to TCR stimulation. It can be seen that naïve CD4⁺ T-cells from CSA patients do not respond to IL-6 in the same manner of healthy controls with no increase in the level of proliferation after 6 days TCR stimulation, *Figure 56*. Although there is a hint that exposure to IL-6 or prior activation in vivo may “blunt” the responsiveness of cells to IL-6 (smaller percent divided in healthy donors compared to other groups), meaning CSA CD4⁺ T-cells have different response to IL-6 compared to healthy control cells, no firm conclusions can be drawn from this limited dataset.

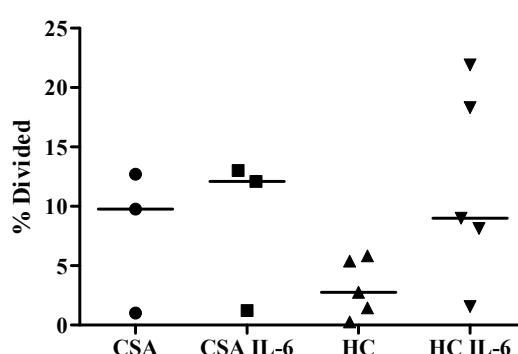


Figure 56. Effect of IL-6 pre-exposure on CSA naïve CD4⁺ T-cells. Naïve CD4⁺ T-cells isolated from 3 CSA patients and 5 healthy controls (HC) were CFSE labelled cultured for 3 days in absence or presence of 0.5ng/ml IL-6 with equimolar sIL-6R. After 72 hours cells were washed and stimulated for 6 days with 0.2µg/ml anti-CD3 and 1µg/ml anti-CD28. After 6 days cells were harvested and ran on the flow cytometer. Proliferation was calculated using FlowJo with percentage divided indicating the precursor frequency of dividing cells. Values were plotted with median values indicated by the horizontal line. Significance was determined by non-parametric analysis of variance (Kruskal-Wallis), $p < 0.05$.

In summary, based on the data presented, CD4⁺ T-cells from early RA patients appear to have a similar ability to proliferate as healthy controls. Furthermore, the level of serum IL-6 in these patients showed no correlation with level of proliferation.

6.3.3 Basal gene expression of early arthritis clinic attendees

In *Chapter 5.3.3*, a number of genes were determined to be differentially expressed between untreated and IL-6 pre-exposed naïve CD4⁺ T-cells 72 hours post-IL-6 exposure. As early RA patients are hypothesised to have already been chronically pre-exposed to high levels of IL-6 in serum it was proposed that levels of expression of the genes found to be differentially

induced/repressed by IL-6 would be up- or down- regulated in rheumatoid arthritis patients respectively compared to healthy controls.

In order to assess whether this is the case CD4⁺ T-cells were isolated from 5 CSA patients, 5 early RA patients, 5 established RA patients and 5 healthy controls and RNA extracted at baseline. As a positive control, RNA was extracted from naïve CD4⁺ T-cells pre-exposed to 0.5ng/ml IL-6 for 72 hours, to confirm that the selection of these genes were valid. From the list of 267 genes identified as differentially expressed following 72 hours exposure to IL-6, 5 genes were chosen to be assessed in disease subgroups. These genes were chosen as they had the high log fold changes but also had strong p values and included BCL6, SBNO2, REXO2, LRP8 and c10orf10. The dynamic profiles of these genes highlight their strong induction by IL-6 after 72 hours IL-6 pre-exposure, *Figure 57*.

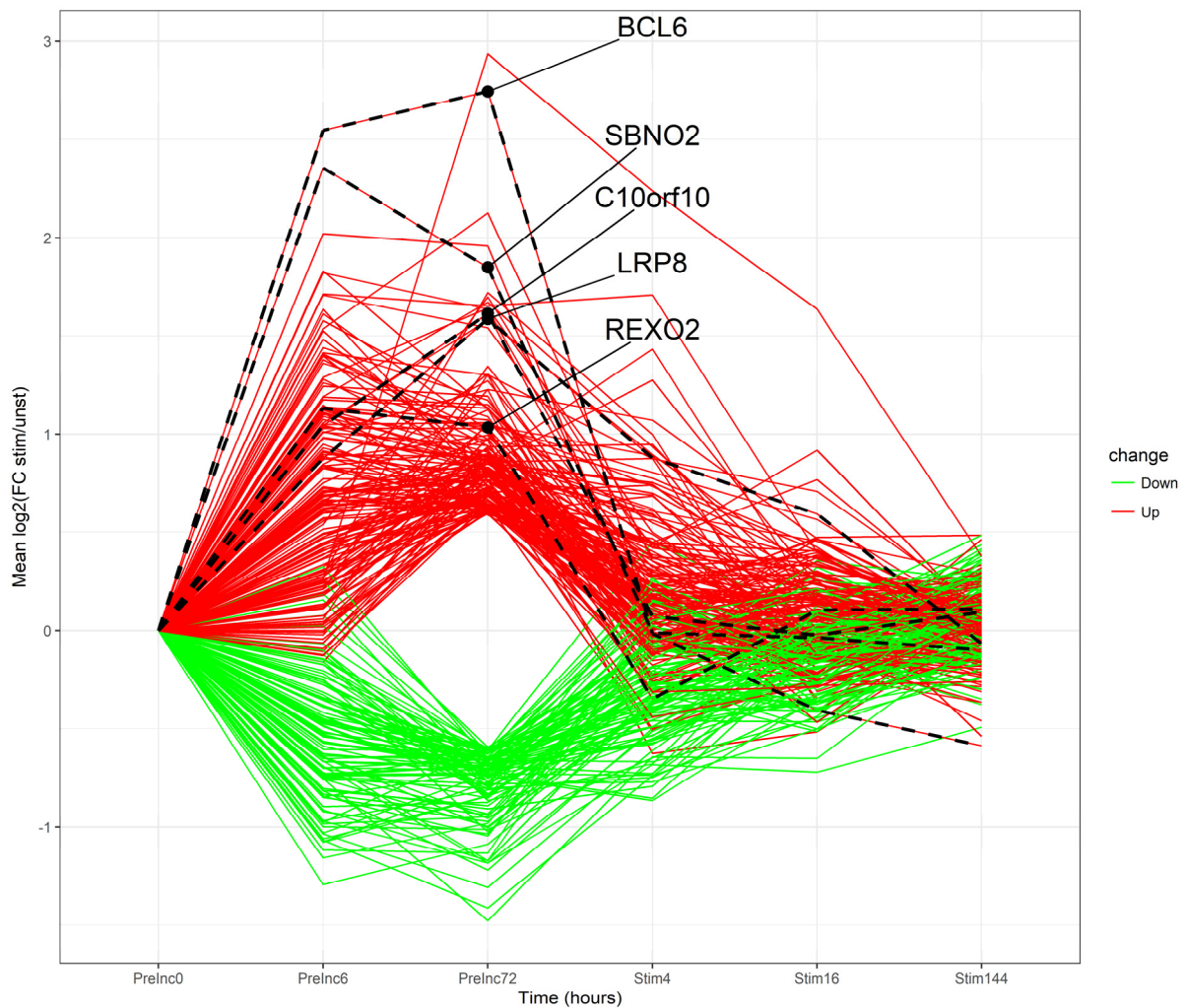


Figure 57. Dynamic profiles of differentially expressed IL-6 pre-TCR signature genes after 72 hours exposure to IL-6. Differentially expressed genes were identified post multiple test correction by cross sectional analysis of untreated and IL-6 exposed naïve CD4⁺ T-cells following 72 hours exposure to 0.5ng/ml IL-6 using paired t-test with LFC 1.5 and p-value cut off of <0.05. Graph shows the five IL-6 pre-TCR gene signature chosen in *Chapter 5* based on strong p-value and high log fold change. Graph representative of three donors.

Expression of all genes were significantly induced in healthy donors with IL-6 exposure compared to non-exposure, *Figure 58A*, as depicted in *Figure 47B*. There was a trend for increased expression of BCL6, SBNO2, REXO2 and c10orf10 in early RA compared to healthy controls but only BCL6 and REXO2 reached statistical significance. Expression levels were similar for all genes between CSA patients and healthy controls whereas expression levels in established RA appeared higher than healthy controls for BCL6, SBNO2 and c10orf10 but lower than healthy controls for LRP8 and no change with REXO2.

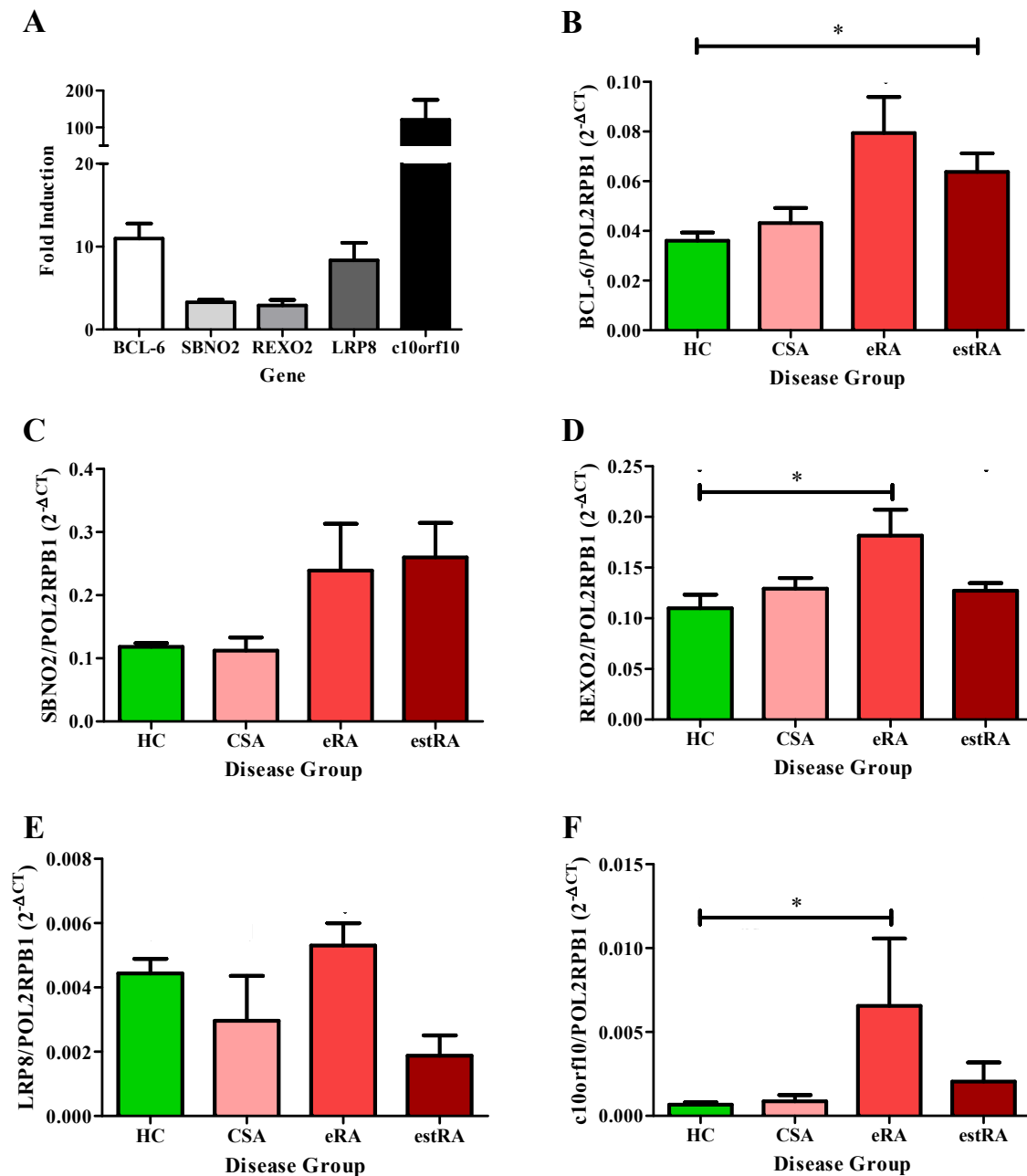


Figure 58. Expression of 72 hour IL-6 induced genes in healthy controls and EAC patient CD4⁺ T-cells. CD4⁺ T-cells were isolated from 5 CSA patients, 5 early RA patients (eRA), 5 established RA patients (estRA) and 5 healthy controls (HC) and RNA extracted at baseline. For a positive control, naïve CD4⁺ T-cells were cultured with 0.5ng/ml IL-6 and equimolar sIL-6R for 72 hours and RNA extracted. (A) Fold induction of each gene with IL-6 stimulation. Expression of BCL6 (B), SBNO2 (C), REXO2 (D), LRP8 (E) and c10orf10 (F) were assessed using TaqMan real time PCR. Values were plotted with bars showing mean and error bars representing SEM. Non-parametric analysis of variance (Kruskal-Wallis) showed significant effect in gene expression irrespective of disease group (*p<0.05 in each case). Comparisons of healthy controls with arthritis disease groups were undertaken as depicted (ns; not significant; *p<0.05, Dunn's post hoc pairwise analysis).

As the genes shown in *Figure 58* were those genes found to be differentially expressed at 72 hours with IL-6 exposure in naïve CD4⁺ T-cells (*Chapter 5*), the level of IL-6 in the serum would be expected to correlate with the expression of these genes. To investigate whether this was the case the level of IL-6 in serum of patients was determined and plotted against gene expression, *Figure 59*. Although there was a trend for a positive correlation between serum IL-6 levels and gene expression for all of the genes analysed this correlation only reaches statistical significance for BCL6 and SBNO2. This suggests that the expression of these genes in CD4⁺ T-cells of CSA, early RA and established RA patients may be driven by the presence of IL-6.

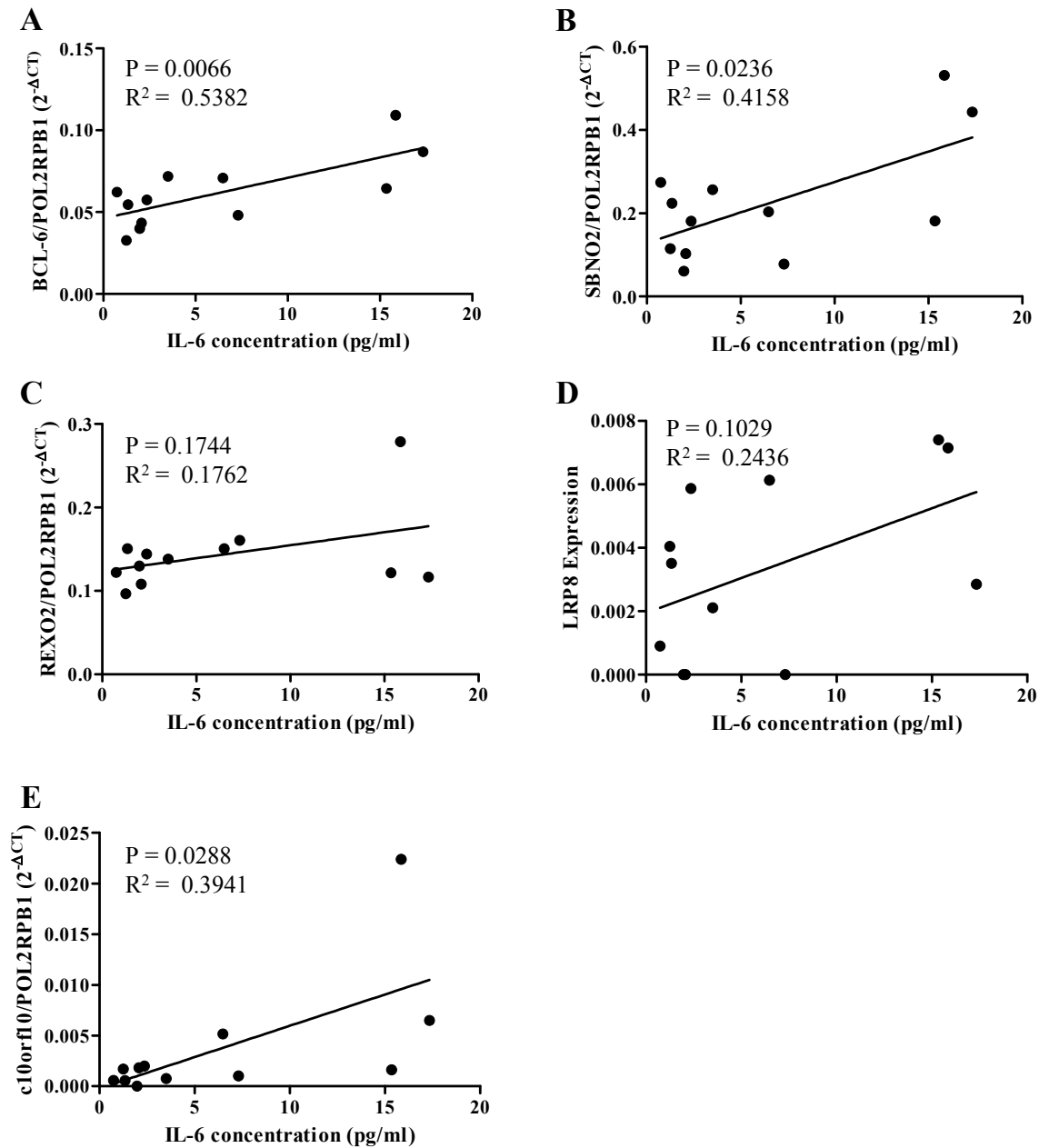


Figure 59. Expression of 72 hour IL-6 induced genes in EAC patient CD4⁺ T-cells correlates with serum IL-6. CD4⁺ T-cells were isolated from CSA patients, early RA patients, established RA patients and healthy controls and RNA extracted at baseline. Serum IL-6 from each patient was measured by MSD and correlated with expression of BCL6 (**A**), SBNO2 (**B**), REXO2 (**C**), LRP8 (**D**) and c10orf10 (**E**) which were assessed using TaqMan real time PCR. Significance was determined by linear regression; * $p < 0.05$. BCL6: $r^2 = 0.5382$, SBNO2: $r^2 = 0.4158$, REXO2: $r^2 = 0.1762$, LRP8: $r^2 = 0.2436$, c10orf10: $r^2 = 0.3941$. CSA $n=5$, eRA $n=5$, estRA $n=5$.

6.3.4 Gene expression of early arthritis clinic attendees after 4-hour TCR stimulation

In *Chapter 5.3.4* there were 123 genes differentially expressed between untreated and IL-6 pre-exposed naïve CD4⁺ T-cells following 4 hours TCR stimulation. To assess whether these differentially expressed genes were also differentially expressed between RA patients compared to healthy controls, five genes from the list of 123 were chosen. The genes chosen include CST7, GPT2 and RRAD as these had the highest induction in terms of log fold change and strongest p values. In contrast, GSTO1 was chosen as this was one of the most differentially expressed genes according to IL-6 pre-exposure, however, in contrast to the other genes this gene was strongly repressed by IL-6 pre-exposure. The dynamic profiles of these genes highlight their strong induction by IL-6 after 72 hours IL-6 pre-exposure, *Figure 60*. Naïve CD4⁺ T-cells were isolated from 5 CSA patients, 5 early RA patients, 5 established RA patients and 5 healthy controls. As a positive control, naïve CD4⁺ T-cells were pre-exposed to 0.5ng/ml IL-6 for 72 hours. All naïve CD4⁺ T-cells were then stimulated with 0.2µg/ml anti-CD3 and 1µg/ml anti-CD28 and RNA extracted after 4 hours stimulation.

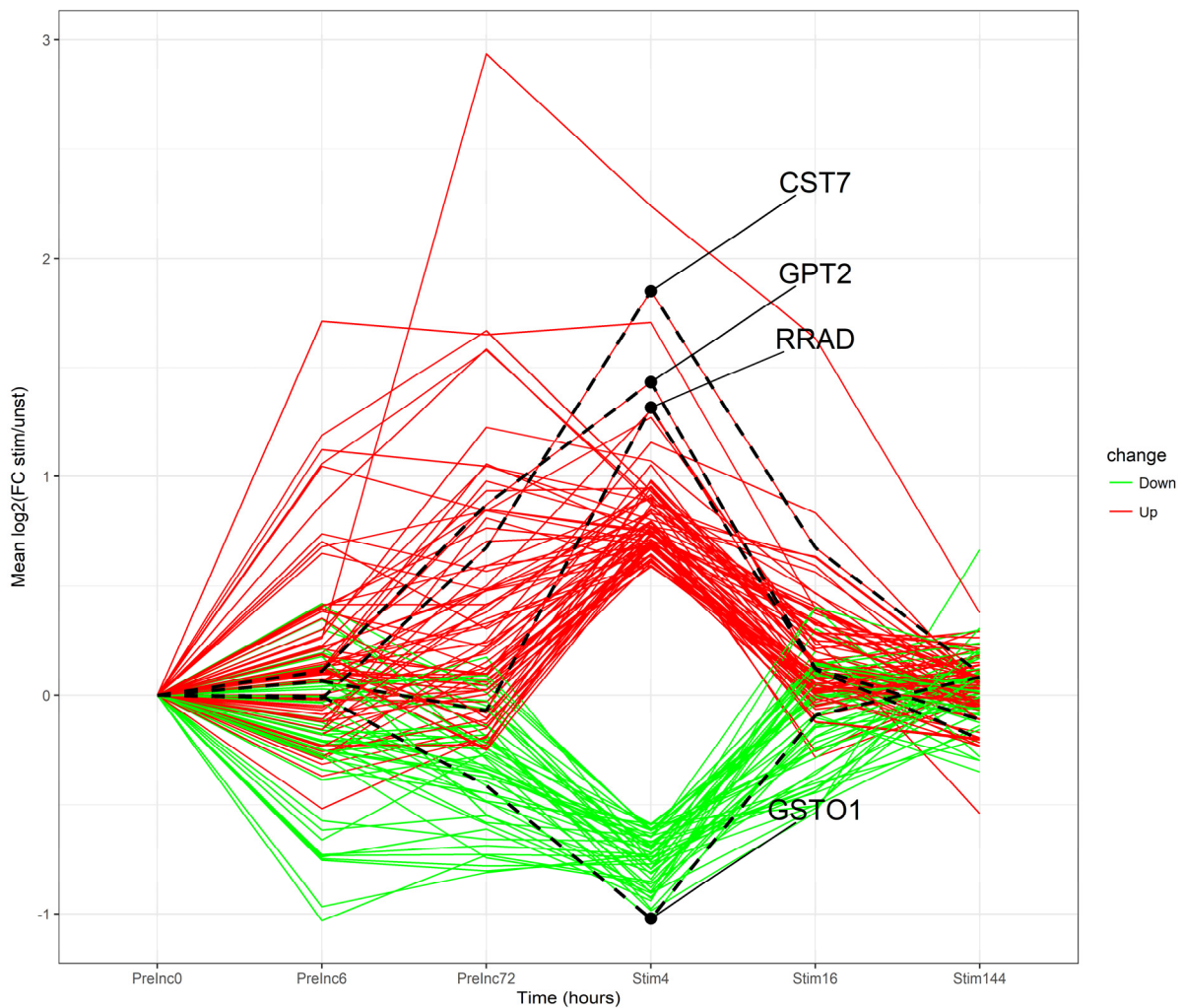


Figure 60. Dynamic profiles of differentially expressed IL-6 post-TCR signature genes after 4-hour TCR stimulation. Differentially expressed genes were identified post multiple test correction by cross sectional analysis of untreated and IL-6 pre-exposed naïve CD4⁺ T-cells following 72 hours pre-exposure to 0.5ng/ml IL-6 and subsequent 4 hours TCR stimulation using paired t-test with log fold change 1.5 and p-value cut off of <0.05. Graph shows the four IL-6 post-TCR gene signature chosen in *Chapter 5* based on strong p-value and high log fold change. Graph representative of three donors.

There was one early RA donor with abnormally high CT values for the housekeeping gene POL2RPB1, which resulted in altered expression of all genes and as a result, this individual was removed from the analysis.

The pre-exposure of naïve CD4⁺ T-cells with 0.5ng/ml IL-6 for 72 hours prior to 4 hours TCR stimulation resulted in the increased induction of CST7 and GPT2 compared to healthy controls, however, IL-6 pre-exposure had no effect on expression of RRAD with similar levels as untreated healthy controls, *Figure 61A*, as also depicted in *Figure 48B*. This is inconsistent with the microarray data in *Chapter 5.3.4*, in which RRAD had strong induction following IL-6 pre-

exposure. GSTO1, which was strongly repressed by IL-6 pre-exposure in *Chapter 5.3.4*, also appeared to have not been altered in response to IL-6 pre-exposure.

Furthermore, there was no clear difference in expression of CST7 or GPT2 between disease groups or healthy controls. There appeared to be a trend for highest expression of RRAD in CSA patients, followed by early RA with lower levels in established RA and healthy controls, although this trend was not statistically significant. As GSTO1 was revealed from the microarray as being down regulated as a consequence of IL-6 pre-exposure it would be expected to be decreased in early RA compared to healthy controls as circulating serum IL-6 levels are increased in early RA compared to healthy controls. However, there is no change in expression across disease groups or between healthy controls and it does not appear to decrease with IL-6 pre-exposure. Furthermore, the serum concentrations of IL-6 from these patients did not correlate with expression of these genes, data not shown.

To summarise 2 of the 4 genes found to be differentially expressed as a result of IL-6 pre-exposure following 4 hour stimulation in the microarray experiment shown in *Chapter 5* were found to be differentially expressed between healthy controls and IL-6 pre-exposed healthy controls using a TaqMan validation assay. However, none of the genes assessed appeared to differ in expression between disease groups or healthy controls.

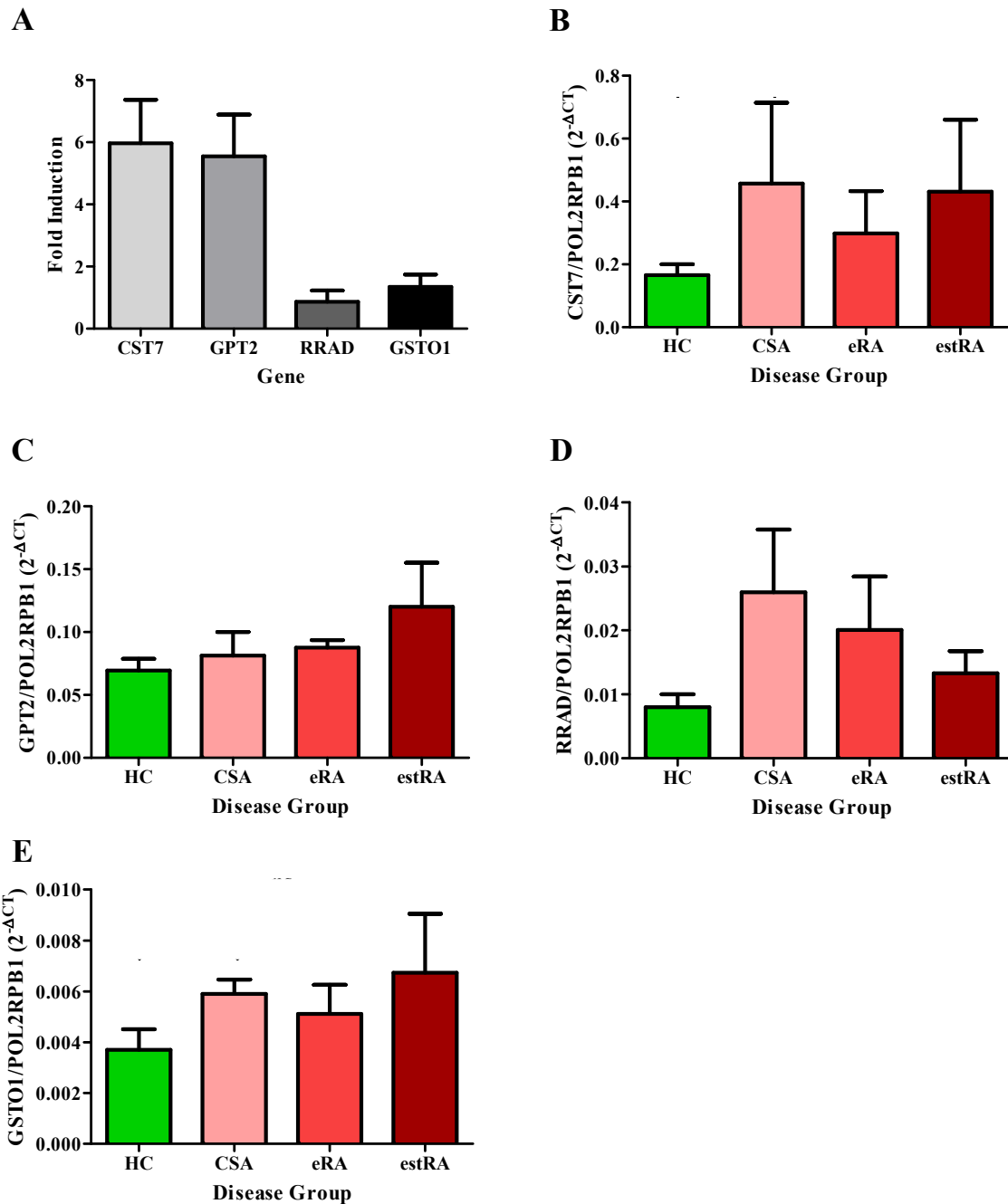


Figure 61. Expression of genes differentially expressed following IL-6 pre-exposure and 4-hour TCR stimulation in healthy controls and EAC patient naïve CD4⁺ T-cells. Naïve CD4⁺ T-cells were isolated from 5 CSA patients, 5 early RA patients (eRA), 5 established RA patients (estRA) and 5 healthy controls (HC). Cells were stimulated with 0.2µg/ml anti-CD3 and 1µg/ml anti-CD28 for 4 hours and RNA extracted. For a positive control naïve CD4⁺ T-cells were cultured with 0.5ng/ml IL-6 and equimolar sIL-6R for 72 hours followed by 4 hour stimulation with 0.2µg/ml anti-CD3 and 1µg/ml anti-CD28 and RNA extracted. (A) Fold induction of each gene with IL-6 stimulation. Expression of CST7 (B), GPT2 (C), RRAD (D) and GSTO1 (E) were assessed using TaqMan real time PCR. Values were plotted with bars representing mean and error bars representing SEM. Non-parametric analysis of variance (Kruskal-Wallis) showed no significant effect in differences in gene expression between the groups. Comparisons of healthy controls with arthritis disease groups were undertaken as depicted (ns; not significant; Dunn's post hoc pairwise analysis).

6.4 Discussion

In this Chapter, the aim of the work carried out was to investigate the potential relevance of the findings in relation to the IL-6 pre-exposure model, presented in previous chapters, to early RA pathogenesis. To do this I explored the extent to which properties of circulating CD4⁺ T-cells from early RA and/or CSA patients mimicked those of healthy donor cells pre-exposed to IL-6 in vitro. CD4⁺ T-cells from early RA patients were assessed in terms of their IL-6 signalling receptors and their response to TCR stimulus at the level of gene expression and proliferative response. This Chapter reveals that early arthritis CD4⁺ T-cells are no different to healthy controls in terms of their gp130 and IL-6R expression, irrespective of disease phenotype, or response to TCR stimulation. In addition, CD4⁺ T-cells from CSA patients appear to have marginally increased levels of proliferation compared to healthy controls, however, they appear to be unable to respond to further IL-6 stimulation. There was increased expression of two genes in early RA CD4⁺ T-cells, which were found to be induced by IL-6 exposure pre-TCR stimulation in vitro; however, there was no difference in expression of genes differentially induced by IL-6 pre-exposure post-TCR stimulation.

6.4.1 Expression of IL-6 signalling receptors in early RA

It was shown in *Chapter 3.3.1* that in CD4⁺ T-cells from EAC patients naïve CD4⁺ T-cells have higher expression of gp130 whereas memory CD4⁺ T-cells have higher expression of IL-6R. This was further confirmed in naïve CD4⁺ T-cells isolated from healthy donors in *Chapter 4.3.1*. This suggests high expression of gp130 on naïve CD4⁺ T-cells is a potential mechanism of increased sensitivity of these cells to IL-6 signalling. Based on these findings it was investigated whether the differential expression of gp130 may account for the STAT-3 gene signature or the high level of pSTAT-3 expression discriminatory for RA in early arthritis.

In this Chapter, no differential expression of IL-6R or gp130 between healthy controls and different disease phenotypes was observed. This observation correlates with previous studies which show that IL-6R expression of CD4⁺ T-cells and serum levels of sgp130 and sIL-6R are equal across disease diagnoses (427). In addition, peripheral blood CD4⁺ T-cells from non-RA inflammatory arthritis patients appear similarly sensitive as early RA patients to circulating IL-6 indicating there is no differential responsiveness of these cells to IL-6 between disease groups. This suggests a mechanism other than differential receptor expression is responsible for the increased pSTAT-3 seen in early RA.

In vivo CRP is a measure of inflammation and correlates with increasing IL-6 levels. As such with increasing CRP decreasing levels of receptor expression would potentially be expected.

However, expression levels of gp130 and IL-6R were found to have no correlation with markers of inflammation, such as CRP.

The presence of CD25 expression on CD4⁺ T-cells has also been shown to correlate with differential expression of IL-6R and gp130 (455). High expression of IL-6R and gp130 has been seen in CD25⁺CD4⁺ T-cells and the increased expression of CD25 has been shown to correlate with the downregulation of expression of gp130. In this Chapter it was seen that with higher expression of CD25 there was no difference in the expression of gp130. In this thesis, CD25 was used as a marker of activation rather than a marker of Treg presence hence may represent a decrease in IL-6 signalling with increasing levels of cell activation. However, in the case of Tregs this downregulation of IL-6 signalling may be important for the maintenance of these cells as IL-6 signalling has been shown to abrogate Foxp3. It has also been described that IL-6 signalling results in the resistance of CD25⁺CD4⁺ effector T-cells to Treg mediated suppression a potential pathogenic mechanism of IL-6, which may also be mediated by IL-6 pre-exposure.

To summarise, there is no difference in IL-6 signalling receptors between early RA and healthy or disease controls. Furthermore, there is no correlation between expression of these receptors with inflammatory markers or CD25 expression.

6.4.2 Proliferation of early RA CD4⁺ T-cells

There is much evidence supporting the dysregulation of CD4⁺ T-cells in RA. The repertoire of naïve and memory CD4⁺ T-cells is markedly contracted in RA compared to healthy controls indicating an abnormality in repertoire formation (525). In addition to the less diverse repertoire RA patients also have fewer T-cell receptor excision circles (TREC) which correlated inversely with inflammation, demonstrating decreased influx of newly generated T-cells or increased cell turnover (107, 108). A decrease in telomere length, most prominent in naïve CD4⁺ T-cells also suggests increased self-replication which occurs very early in disease, even possibly pre-dating disease onset although this does not correlate with disease duration (107). These findings suggest that homeostatic proliferation of CD4⁺ T-cells is dysregulated in RA. Further evidence suggests in healthy individuals homeostatic proliferation requires MHC II whereas in RA this is not the case and is dependent on membrane bound TNF- α (526).

Despite the increased self-replication suggested by shortened telomeres, many studies have documented the impaired proliferative response of RA CD4⁺ T-cells. This has been shown by the reduced proliferation of CD4⁺ T-cells with active RA in culture with autologous MLR though proliferation can be partially restored by IL-2 (112, 113). CD4⁺ T-cells from patients

with RA stimulated with PHA or anti-CD3 also resulted in a larger number of non-responsive cells and impaired magnitude of response (111). Further investigation reveals cell cycle duration, number of cells dividing and number of cell divisions are all reduced in RA whereas the time to transition from G0 to G1 was increased (114). As well as reduced proliferation RA CD4⁺ T-cells have been shown to have increased CD25, PD-1, CTLA-4 but decreased CD69, OX40 and IL-2 expression (520). These features are indicative of an exhausted phenotype and immunosenescence of CD4⁺ T-cells in RA.

The impaired proliferative response of RA CD4⁺ T-cells conflicts with the data presented in this chapter, which show similar levels of proliferation compared to healthy controls, *Figure 54*. The difference in proliferation seen may be due to the use of different cell populations or difference of patient groups used for the study of proliferation. For example, many of these papers involved the stimulation of CD4⁺ T-cells in a PBMC culture and the presence of other cells in this system may be contributing to the suppression of CD4⁺ T-cell proliferation. In this Chapter, total CD4⁺ T-cells were purified prior to anti-CD3 and anti-CD28 stimulation which limits the number of confounding factors in this system. A number of papers also involved the recruitment of patients presenting with arthritis flare and patients on a range of immunomodulatory treatments. This contrasts with the patients used in this Chapter, which were drug naïve early RA patients, which may explain the difference of the ability of CD4⁺ T-cells to proliferate.

The similar levels of proliferation of early RA patients and healthy controls is contrary to the effect predicted as a result of IL-6 pre-exposure evidenced by increased proliferation, *Chapter 4.3.3*. One explanation for this is that the IL-6 stimulus used in the in vitro model is much greater than the levels seen in disease. Although this was optimised in *Chapter 4* to be the concentration which was found at physiologically relevant levels to disease while still resulting in differences to effector phenotype this concentration may not reflect actual physiological functional levels and the levels of IL-6 in the serum of these patients was found not to correlate with the level of proliferation of CD4⁺ T-cells. This may be because these patients have already developed active disease so have high levels of inflammation and a range of other cytokines may be present. Another reason for this may be due to the high levels of sgp130 found in the serum of early RA patients, which acts as an inhibitor of the IL-6 signalling pathway. Despite 100-500pg/ml being physiologically measurable, it may be that once the IL-6-sIL-6R complex binds to sgp130 this pathway is turned off resulting in functionally lower levels. In addition, the CD4⁺ T-cells from these patients will have already encountered antigen in vivo hence will have already undergone activation. Furthermore, work in Chapter 4 was carried using naïve

CD4⁺ T-cells whereas in early RA patients total CD4⁺ T-cells were used for measurement of proliferation. Due to the differences in sensitivity to IL-6 seen between naïve and memory CD4⁺ T-cells differences in response to IL-6 between naïve and total CD4⁺ T-cells would be expected which may in part explain the lack of difference seen between early RA and healthy controls.

The limited number of early RA patients recruited for proliferation analysis in this Chapter mean this data has low statistical power with the possibility of overestimating effect size meaning that it is not possible to draw firm conclusions regarding the proliferative capacity of early RA CD4⁺ T-cells (527). For this data to have more statistical power more patients would need to be recruited which was not possible in the time frame of this project. Analysis of ex vivo CD4⁺ T-cells carried out previously with the group (Amy Anderson, unpublished data). show increased expression of ki-67 in RA CD4⁺ T-cells compared to healthy controls further indicating increased CD4⁺ T-cell proliferation in early RA, *Figure 62*. This increase in ki67 expression in ex vivo CD4⁺ T-cells from early RA patients would suggest CD4⁺ T-cells from early RA do have increased proliferative capacity which may result from pre-exposure to IL-6 in vivo.

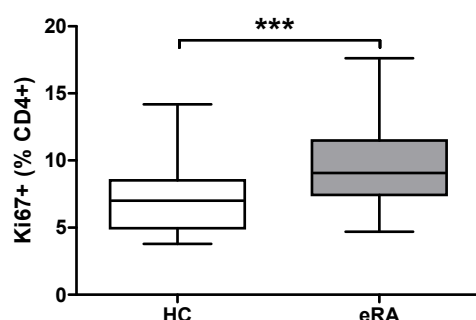


Figure 62. Early RA patient CD4⁺ T-cells express higher ki67 than healthy controls.

PBMC were isolated from peripheral blood of healthy controls (HC) and early drug-naïve RA patients (eRA). Ki67 expression in CD4⁺ T-cells was assessed by flow cytometry. *** p<0.001 calculated with Mann Whitney test. HC n = 48; eRA n = 25. Figure from Dr Amy Anderson, Newcastle University.

Finally, CSA patients, representing the “pre-RA” state appear to have a trend for higher percentage of dividing cells and have slightly higher number of divisions. However, as previously mentioned this only represents a limited number of patients. Due to the rarity of these patients presenting in the clinic the recruitment of these patients was slow and ideally more patients would be recruited for further investigation.

The reason for a trend for increased CD4⁺ T-cell proliferation in CSA and not in early RA compared to healthy controls, may be that CSA patients have not developed as severe joint inflammation. At this pre-RA stage there may be less other pro-inflammatory cytokines present and cells may be in a less activated state meaning the effects of IL-6 can be more closely seen. Another reason for the difference in proliferation between CSA and early RA patients may be due to the different cell types used. As in the in vitro model naïve CD4⁺ T-cells were used for analysis of the proliferation in CSA patients. These cells will be undergoing primary stimulation compared to early RA CD4⁺ T-cells which contain previously in vivo stimulated memory CD4⁺ T-cells. The restimulation of these memory CD4⁺ T-cells may account for their reduced proliferation compared to naïve CD4⁺ T-cells of CSA patients. Further characterisation of CSA and early RA CD4⁺ T-cell proliferation is required to further elucidate the effect of IL-6 in these cells.

When CSA naïve CD4⁺ T-cells pre-exposed to IL-6 for 3 days were TCR stimulated there was only limited effect of IL-6 on the levels of cell proliferation. One possible explanation for this is that these patients may have higher basal levels of IL-6 which will serve as the “pre-exposure” stage. Another potential reason for the unresponsiveness of these cells to IL-6 pre-exposure is their high level of activation. Immediately following TCR stimulation cells have decreased expression of IL-6R and gp130 and the decrease in expression of these receptors may explain their apparent insensitivity to further IL-6 stimulation, however, the expression of these receptors on CSA CD4⁺ T-cells was not measured. The levels of IL-6 in the serum of these patients was assessed and a trend was seen for increasing concentrations of IL-6 with higher levels of proliferation of naïve CD4⁺ T-cells. This mirrors the effect of IL-6 pre-exposure on naïve CD4⁺ T-cell proliferation in the in vitro model (*Chapter 4*).

6.4.3 IL-6 mediated gene expression in early RA CD4⁺ T-cells

In this Chapter it was seen that basal CD4⁺ T-cells isolated from early RA patients had increased expression of a number of genes up-regulated by prolonged exposure to IL-6 identified using an in vitro model (*Chapter 5*). Early RA patients have been shown to have differential expression of STAT-3 regulated genes which correlate with serum IL-6 levels (431). The presence of these chronic IL-6 regulated genes further suggests high levels of IL-6 in the circulation of these patients mediating differential gene expression. It was also seen that there was a trend for the increased expression of certain genes differentially expressed as a consequence of IL-6 pre-exposure followed by TCR stimulation although this did not reach significance.

The genes that were chosen for real-time PCR analysis were chosen on the basis of log fold change and p value. However, there were 267 genes differentially expressed in naïve CD4⁺ T-cells following 72-hours chronic exposure to IL-6 compared to the untreated condition and 123 genes differentially expressed following IL-6 pre-exposure and then 4-hours TCR stimulation compared to non-pre-exposed cells. Time restrictions meant more genes could not be further investigated, however, it is possible that many more genes may be differentially expressed in early RA compared to healthy controls both pre- and post- TCR stimulation and this would be an interesting field of further investigation.

All genes investigated involved the culture of healthy controls with 0.5ng/ml IL-6 with or without TCR stimulation as a positive control to confirm that expression of these genes are altered as a consequence of IL-6 pre-exposure providing technical validation of the system. All genes were significantly induced by 72 hours culture with IL-6 compared to untreated healthy controls suggesting a direct role for IL-6 in expression of these genes, *Figure 58*.

Genes found in *Chapter 5.3.4* to be differentially expressed by prolonged IL-6 exposure were found to show a trend for increased expression in early RA patients compared to healthy controls, with two of these genes reaching statistical significance. On the other hand expression of these genes in CSA patients were similar to that of healthy controls which was unexpected as changes in gene expression would be expected to correlate with the enhanced level of proliferation seen. Expression levels of these genes in established RA vary and are generally higher than healthy controls but lower than early RA, indicating treatment may alter IL-6 levels. These results conflict with proliferation which was seen to be similar to healthy controls in early RA CD4⁺ T-cells but increased in CSA patients compared to controls. This suggests that the genes selected for validation were not directly relevant to proliferative response of CD4⁺ T-cells.

In contrast, no strong conclusions can be drawn from the expression of genes induced 4 hours post-TCR stimulation. As the positive control for this experiment, IL-6 pre-exposed naïve CD4⁺ T-cells followed by 4 hour TCR stimulation, only resulted in the differential expression of only 2 out of the 4 genes shown to be differentially expressed 4 hours post-TCR stimulation with IL-6 pre-exposure, this experiment requires further validation, *Figure 61*.

This in vitro system is a reductionist approach to a highly complex in vivo situation and assumes a prior IL-6 pre-exposure period is occurring in vivo, which needs to be confirmed by analysis of IL-6 levels in serum. Realistically the in vivo situation is much more complex with many other cytokines and other soluble mediators, such as sgp130, which may alter the ability of IL-

6 to induce subsequent gene expression following TCR stimulation. More optimisation of this complex model would be required in order to delineate the effects of IL-6 pre-exposure on early RA gene expression. Alternatively, sampling the population at baseline and subsequently 4 hours post-TCR stimulation to assess the expression of genes at each time-point would provide the relative induction of each gene for both the untreated and IL-6 pre-exposed cells. This analysis would take into account potential differences in basal gene expression, which may be different in early RA compared to healthy controls, despite similar levels of final expression.

To summarise early RA patients have increased expression of a number of genes identified following prolonged exposure to IL-6 and potential induction of genes differentially induced post-TCR stimulation highlighting IL-6 pre-exposure as an event in early RA.

6.4.4 Summary

In conclusion, this Chapter demonstrates that early RA patients have high levels of expression of a number of genes shown to be differentially expressed as a result of IL-6 pre-exposure in vitro. This confirms the up-regulation of IL-6 mediated STAT-3 signalling in early RA. Some of the consequences of IL-6 pre-exposure on healthy control naïve and memory CD4⁺ T-cells can be seen to be reflected in the phenotype of early RA CD4⁺ T-cells, such as increased Ki67 and increased expression of IL-6 induced genes. This is consistent with an important role for IL-6 mediated STAT-3 signalling early in the pathogenesis of RA.

Chapter 7. General discussion and future work

The work outlined in this thesis extends upon previous findings which highlight IL-6 mediated STAT-3 signalling as an important event in the earliest stages of RA pathogenesis; which, through transcriptional imprinting may programme aberrant effector responses of naïve and/or memory CD4⁺ T-cells (427, 431).

7.1 Differential expression of IL-6 signalling receptors

It was previously suggested that naïve CD4⁺ T-cells were more sensitive to circulating IL-6 than their memory CD4⁺ T-cell counterparts, evidenced by their strong correlation between circulating IL-6 levels and activated phosphorylated STAT-3 (427). The assessment of expression of IL-6 receptors on CD4⁺ T-cell subsets revealed maximal expression of the β subunit, signalling receptor gp130 on naïve CD4⁺ T-cells compared to both central and effector memory CD4⁺ T-cells. In contrast, IL-6R, the α subunit, was shown to be lowest on naïve CD4⁺ T-cells and highest on central memory CD4⁺ T-cells, those which display reduced sensitivity to IL-6. These observations suggest that expression of IL-6 receptor subunits could be linked to the antigen experience of the T-cell, providing further insight to previous descriptions of receptor expression (368, 528).

As previously described the sIL-6R can be found in excess of IL-6 in circulation, therefore increased expression of gp130 of naïve CD4⁺ T-cells provides a potential mechanism for increased IL-6 trans signalling (rather than classical signalling) as the mediator of STAT-3 signalling in these cells. The trans signalling pathway of IL-6 has been linked to the pathogenic features of the IL-6 signalling pathway and has been shown to play an important role in the development of numerous immune-mediated inflammatory diseases, suggesting that in early RA naïve CD4⁺ T-cells may be driving pathogenic cell types with consequences relevant to RA pathogenesis (291, 456, 529).

The absence of sIL-6R during IL-6 pre-exposure resulting in similar levels of proliferation following TCR stimulation, hamper the hypothesis that IL-6 pre-exposure is mediated by IL-6 trans signalling. Therefore further investigation into the contributions of classical and trans signalling, by blocking of membrane bound IL-6R or partial blocking of gp130, in the effects of IL-6 exposure should be further explored.

7.2 IL-6 pre-exposure results in pathogenic phenotype

The presence of IL-6 and also its presence *during* activation has been shown to mediate effects such as decreased cell death, enhanced proliferation and numerous consequences on T-helper

cell differentiation (530). This is the first study to show that cytokine “priming” of naïve CD4⁺ T-cells in the circulation, often predating the clinical onset of RA, has the ability to programme hyper-proliferation, activation and a propensity towards Th1 differentiation upon subsequent antigen encounter, even when this occurs in the tissue where IL-6 may no longer dominate (531). Despite the known effect of IL-6 on cell survival, an influence of IL-6 pre-exposure on cell viability was not observed, *Chapter 4.3.1*, suggesting the effect of this cytokine on cell survival requires the presence of IL-6.

Increased proliferation seen with IL-6 pre-exposure may contribute to the hyper-proliferative phenotype of CD4⁺ T-cells in early RA leading to accumulation of cells within the joint. Increased activation of these cells may contribute to the recruitment and activation of further immune cell subsets. The increased production of IFN- γ from Th1 cells may drive the activation of inflammatory M1 macrophages which further drive inflammation and joint destruction. The effects of IL-6 pre-exposure on T-helper differentiation opposes the reported roles of IL-6 upon T-helper differentiation however these may depend upon continuous availability of IL-6 during stimulation and the presence of other mediators such as TGF- β (364, 474, 532). Despite the debate surrounding these cytokines in the involvement of RA, IL-6 pre-exposure may potentially alter the balance of these subsets with important implications for disease progression.

7.3 IL-6 pre-exposure reveals significant changes in gene expression

The observation of increased sensitivity of naïve CD4⁺ T-cells was further confirmed at the transcriptional level. Furthermore, the transcriptional profile of IL-6 exposed naïve CD4⁺ T-cells showed striking similarity to those genes previously observed to be differentially expressed between early RA and non-RA patients, providing strong evidence that IL-6 is a key driver of this gene signature in early RA (427, 431). Furthermore, the genes found to be differentially expressed post-TCR stimulation in IL-6 pre-exposed naïve CD4⁺ T-cells compared to untreated naïve CD4⁺ T-cells were enriched for those involved in pathways such as lymphocyte proliferation and survival, which reflect the hyper-proliferating effector phenotype of these cells.

The consequences of IL-6 pre-exposure on effector phenotype and gene expression post-TCR stimulation highlight the possibility of cellular pre-programming via cytokine exposure as an important event in the inflammatory process. The prolonged exposure of CD4⁺ T-cells to pro-inflammatory cytokines such as IL-6 may have the ability to cause cellular programming with consequences specifically relevant in the early disease setting. A potential mechanism for this event could be that prolonged exposure to cytokines may result in epigenetic changes, such as

DNA methylation, whereby the expression of certain genes is altered therefore affecting the proteins which are transcribed. This has the ability to unmask potential disease relevant expression quantitative trait loci (eQTLs), therefore influencing disease development or progression.

7.4 Relevance of IL-6 pre-exposure in CD4⁺ T-cells from early RA

No significant differences were seen in the phenotype of early RA CD4⁺ T-cells compared to healthy controls. In contrast, preliminary data from a limited number of CSA patients, who have joint pain but no obvious signs of inflammation, appear to proliferate to a slightly higher level than healthy controls and this level appears to show correlation with serum levels of IL-6. It is possible that the cytokine milieu is more complex in early RA and other circulating cytokines may be confounding the effects of IL-6 pre-exposure (531). However, it is also possible that in early RA the CD4⁺ T-cells have experienced a far longer period of chronic pre-exposure and subsequent activation. These CD4⁺ T-cells in early RA may then in fact be “exhausted” due to the increased level of cycling in vivo, indicated by the increased expression of ki67 in early RA patient CD4⁺ T-cells. This may explain why no difference in proliferative capacity can be seen upon ex vivo stimulation. It may also be the case that this exhaustion has not yet reached its peak in CSA.

The model of IL-6 pre-exposure optimised in this thesis highlights the potential of an inflammatory cytokine, such as IL-6, as a surrogate autoantigen in those pre-disposed to RA, with a particularly prominent role for this mechanism in seronegative RA where disease relevant mechanisms of pathogenesis remain elusive, *Figure 63*. Those individuals genetically pre-disposed to RA, via HLA haplotype or a combination of SNPs, may undergo an environmental triggers such as an infection, resulting in the increased production of IL-6. In certain individuals this increased production of IL-6 may not be regulated, possibly due to SNPs affecting the IL-6 region or the hypo-methylation of the IL-6 promoter. This prolonged period of IL-6 exposure has the potential to programme naïve CD4⁺ T-cells in the periphery, via transcriptional imprinting. Following their migration to lymph nodes or lymphoid follicles in the joint these cells may be activated via the TCR following a second hit/ trigger and these cells have an aberrant effector phenotype where hyper-proliferation, activation and IFN- γ production drive inflammation, immune cell recruitment and activation and joint destruction.

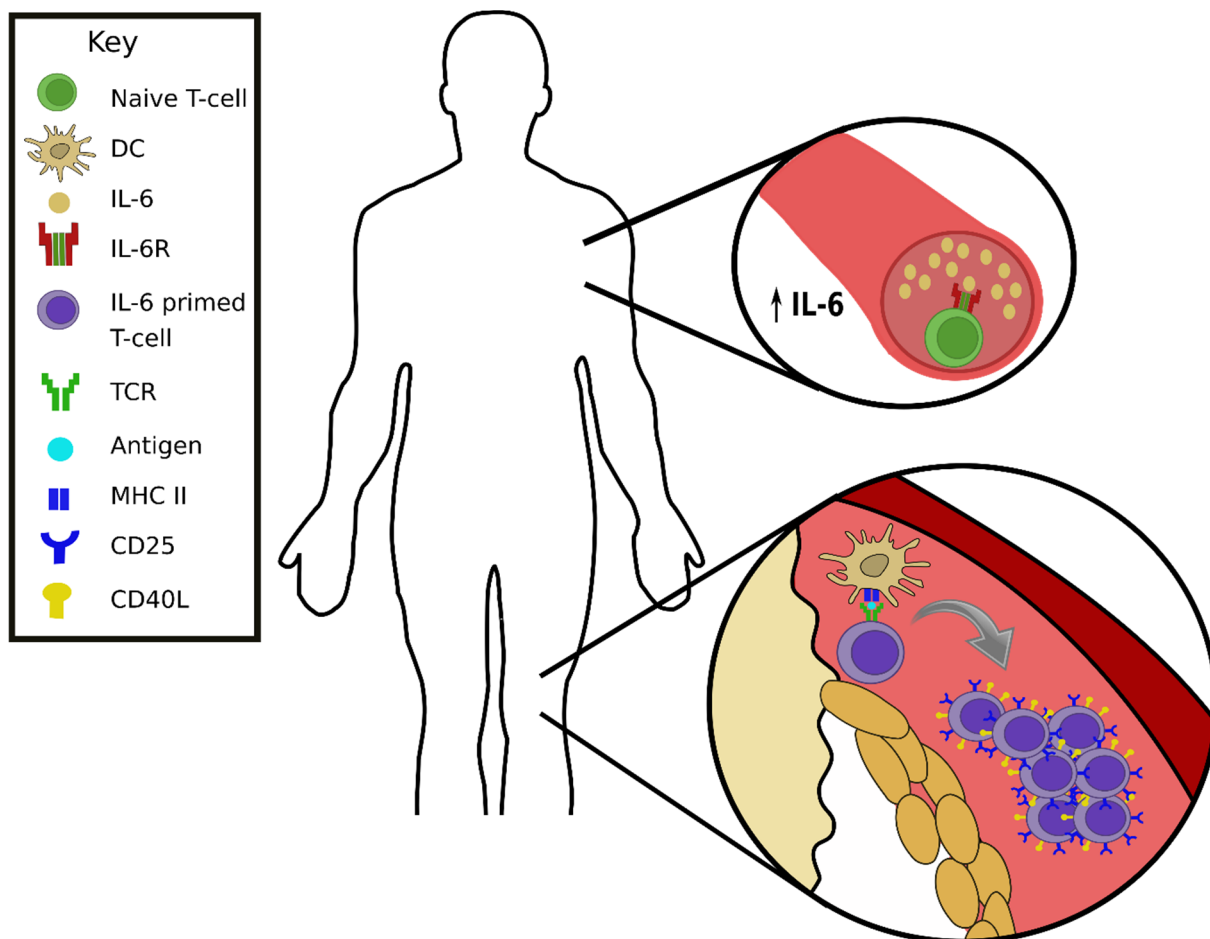


Figure 63. Model of IL-6 pre-exposure in vivo. Chronic exposure to high levels of circulating IL-6, as seen in early RA, has the potential to programme naïve CD4⁺ T-cells in the periphery. These cells will then migrate to the lymph nodes or to lymphoid follicles in the joints where these cells are subsequently stimulated via the TCR. Following stimulation these cells then respond aberrantly with increased proliferation and high levels of activation, such as CD25 and CD40L. Together this has the potential to exacerbate disease, providing a mechanism of antigen independent immune dysregulation.

This model may provide a potential experimental method of treatment stratification. The use of therapies which target IL-6 depend upon its well known role as a pro-inflammatory mediator in established disease (533). It is now being realised that IL-6 has a complex mechanism of signalling and a uniquely important role in early disease and as such this therapeutic strategy should now be reconsidered (221, 534, 535). The proliferation and activation of CD4⁺ T-cells both in absence and presence of IL-6 pre-exposure can be assessed alongside circulating IL-6 levels to identify a particular “endotype” of patients, those for which IL-6 signalling blockade would prove to be maximally effective in treating disease. This provides potential justification for the treatment of such patients, with high circulating IL-6 levels, early in disease and potentially even prior to disease onset, with a biologic therapy such as IL-6 blockade as a first line treatment. This early stage of disease may be the correct phase at which this treatment

should be used. Treatment with biologics at the correct disease stage, prior to joint degradation, means that other pathways in addition to inflammatory pathways will be targeted. For example, temporary IL-6 signal blockade during the preclinical phase of RA, tailored to target the trans signalling pathway, could reverse transcriptional imprinting in a time-dependent manner and alter disease progression (536). The targeting of disease relevant cellular dysregulation in a particular disease endotype provides the potential for a more tolerogenic rather than merely anti-inflammatory effect, with the potential to induce drug free remission.

7.5 Future work

The work outlined in this thesis opens up several areas for further investigation.

7.5.1 Validation of the “post-TCR signature”

Validation of the “4-hour post-TCR gene signature” following IL-6 pre-exposure is necessary before further analysis of these genes in arthritis patients can be carried out. The presence of the “4-hour post-TCR gene expression signature” was determined in *Chapter 6.3.4* by real-time PCR analysis of pre-selected highly differentially expressed genes. Levels of expression of these genes were determined for several stages of disease and compared with healthy controls but no differences in expression of these genes was seen between these groups. Other candidate genes, such as those specifically identified as being involved in proliferative responses, would be worth investigating in these patient groups. To identify these genes for example TaqMan low density array (TLDA) or nanostring could be carried out on CD4⁺ T-cells of subgroups of arthritis patients to determine the expression of all 123 differentially expressed genes found in naïve CD4⁺ T-cells. These are techniques to identify specific genes of interest by the binding to corresponding probes. The expression of these genes could be measured in a number of early RA patients following 4 hour TCR-stimulation to determine the level of overlap of gene expression of early RA compared to IL-6 pre-exposed cells. This list could then be narrowed down to the most differentially expressed genes between early RA and healthy controls to produce an “IL-6 driven post-TCR gene signature”.

7.5.2 Role of IL-6 in CSA

The preliminary data showing a potential correlation of circulating IL-6 levels with levels of proliferation of CSA CD4⁺ T-cells, *Chapter 6.3.2*, highlights the role of IL-6 in the very earliest stages of RA. This is potentially important as it provides possible identification of a disease endotype in which IL-6 signalling is particularly important in disease pathogenesis. This then provides justification for the use of IL-6 targeting therapies in this group of individuals. This finding needs to be confirmed and further investigation is required to investigate the contribution of this mechanism at this early stage of disease.

7.5.3 Potential relevance of IL-2 signalling

IL-2 is a key cytokine in T-cell maintenance which supports proliferation and survival. IL-2 is produced by T-cells in response to TCR stimulation, with levels proportional to the strength of stimulation. IL-6 has also been shown to induce the production of IL-2 from T-cells which can then be used by the cells with rapid and steady turnover. Therefore, the role of IL-2 in the IL-6 pre-exposure model requires further investigation. The consequences of IL-6 pre-exposure may occur as a direct result of IL-6 signalling or may occur indirectly by secondary signalling by IL-2 as IL-2 signalling causes the decreased expression of gp130 to prevent further IL-6 signalling. In order to address this possibility levels of IL-2 in the supernatants of IL-6 pre-exposed naïve CD4⁺ T-cells was assessed in *Chapter 4.3.8*, and it was seen that levels of IL-2 are not increased significantly in the 0.5ng/ml IL-6 pre-exposed cells compared to untreated cells.. Due to the quick turnover of this cytokine and the dependence of CD4⁺ T-cells on this cytokine for survival. The influence of IL-2 in particular upon observations attributed to IL-6 cannot be entirely excluded. The addition of an IL-2 blocking antibody will reveal how dependent the IL-6 pre-exposure model is on the presence of IL-2.

7.5.4 Contributions of classical and trans signalling to the processes of IL-6 pre-exposure

The expression of the gp130 signalling receptor on CD4⁺ T-cell subsets, *Chapter 3.3.1*, and its correlation with sensitivity to IL-6 signalling implicates the trans signalling pathway as the primary mechanism of the IL-6 pre-exposure effect. In contrast, in *Chapter 4.3.3* the contributions of the classical and trans signalling pathways were briefly reviewed in terms of proliferative responses of naïve CD4⁺ T-cells. This revealed that even in the absence of sIL-6R increases in proliferation following IL-6 pre-exposure and subsequent TCR stimulation were still observed. Further work may therefore aim to assess the relative contributions of each of these signalling mechanisms to the consequences of IL-6 pre-exposure This could be assessed experimentally with the use of molecules such as sgp130Fc to specifically block the trans signalling pathway (264).

7.5.5 Effector resistance to Treg suppression

There is mixed evidence as to whether there is impaired function of Treg cells in RA. However, in several inflammatory diseases effector cells have been shown to become resistant to suppression by Tregs. A role for IL-6 mediated STAT-3 signalling has been outlined in these diseases and work highlighting the importance of the STAT-1/STAT-3 balance as a predictive biomarker of early RA further implicates the signalling pathway in early disease. Data presented herein indicates cells pre-exposed to IL-6 display increased levels of proliferation and activation displaying an increased propensity for pro-inflammatory cytokine production upon

differentiation under Th1 skewing conditions. This raises the hypothesis that elevated circulating IL-6 in the earliest phase of RA leads to STAT-3 mediated transcriptional pre-programming of antigen inexperienced naïve CD4⁺ T-cells which, upon TCR stimulation, results in an effector phenotype resistant to Treg suppression. To test this hypothesis, healthy control Teff cells could be pre-exposed to IL-6 followed by co-cultured with allogenic Tregs in a suppression assay to assess the capacity of these cells to be suppressed. The ability of these cells to proliferate as well as expression of activation markers would be assessed to determine their resistance to suppression. It would be of further interest to carry out similar experiments using naïve CD4⁺ T-cells from early RA and tocilizumab treated RA patients to assess the relevance of this mechanism of IL-6 pre-exposure in disease pathogenesis. This may yield insight into a novel mechanism of immune tolerance loss in early RA. These readouts may then be a valuable tool in defining an “endotype” of early RA for those whom IL-6 signalling blockade may prove most effective in treating disease and potentially even inducing drug-free remission.

7.6 Summary

In conclusion a model to test the consequences of cytokine pre-exposure and their ability to pre-programme naïve and memory CD4⁺ T-cells has been developed. These findings indicate that pre-exposure of cells to IL-6 pre-programmes cells to respond aberrantly when faced with subsequent TCR stimulation. This aberrant response includes increased proliferation and activation and increased pro-inflammatory cytokine production on Th1 differentiation, providing a more pathogenic phenotype. My findings require further study, but could have implications both for pathogenic understanding of this heterogeneous disease, and for designing optimal IL-6 targeting strategies for its management.

Appendix

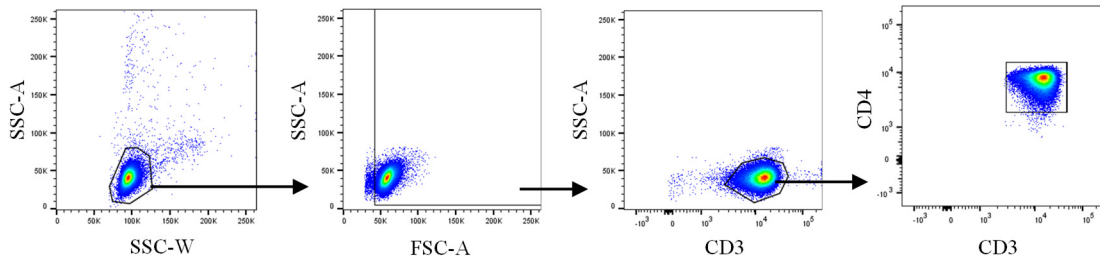


Figure 1. Gating strategy for total CD4⁺ T-cell purity analysis. Total CD4⁺ T-cells were stained with purity panel of antibodies prior to acquisition on the flow cytometer. Cells were gated on SSC-A v SSC-W to exclude debris followed by SSC-A v FSC-A to gate lymphocytes. CD4⁺ T-cells were gated as CD3⁺CD4⁺.

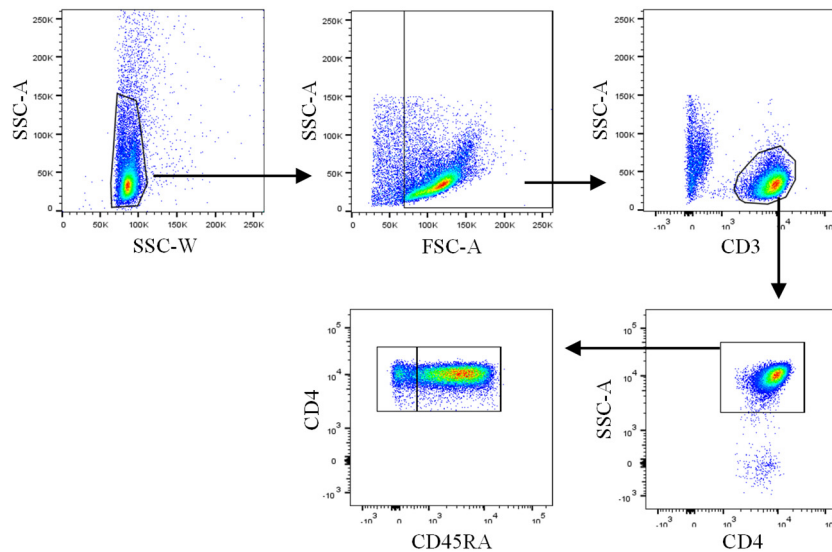


Figure 2. Gating strategy for naïve CD4⁺ T-cell purity analysis. Naïve CD4⁺ T-cells were stained with purity panel of antibodies prior to acquisition on the flow cytometer. Cells were gated on SSC-A v SSC-W to exclude debris followed by SSC-A v FSC-A to gate lymphocytes. CD4⁺ T-cells were gated as CD3⁺CD4⁺ and naïve CD4⁺ T-cells identified as CD4⁺CD45RA⁻. Gates were set based on the expression of CD45RA on whole CD4⁺ T-cells and transferred to naïve and memory populations.

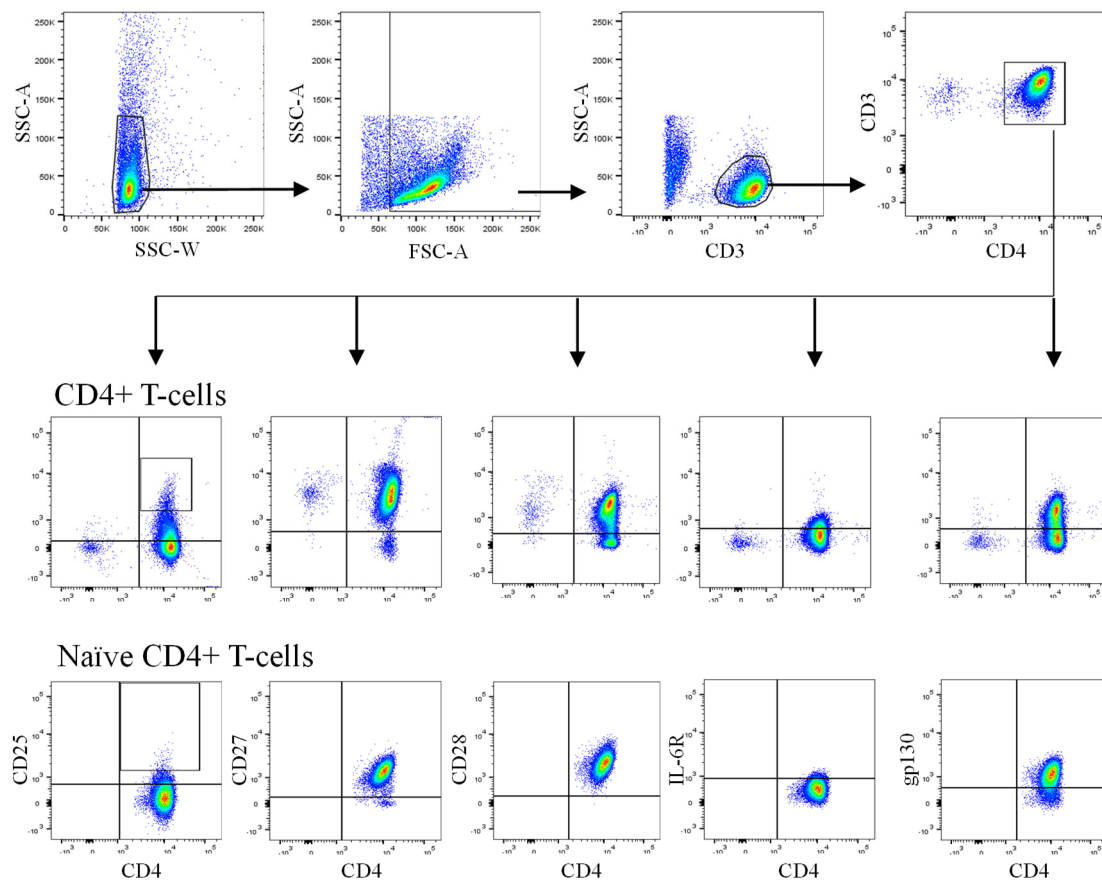


Figure 3. Gating strategy for phenotype analysis of naïve and memory CD4⁺ T-cells. Naïve or memory CD4⁺ T-cells were stained with phenotype panel of antibodies prior to acquisition on the flow cytometer. Cells were gated on SSC-A v SSC-W to exclude debris followed by SSC-A v FSC-A to gate lymphocytes. CD4⁺ T-cells were gated as CD3⁺CD4⁺. Gates of phenotypic markers were set based on the expression of the marker on whole CD4⁺ T-cells and transferred to naïve and memory populations.

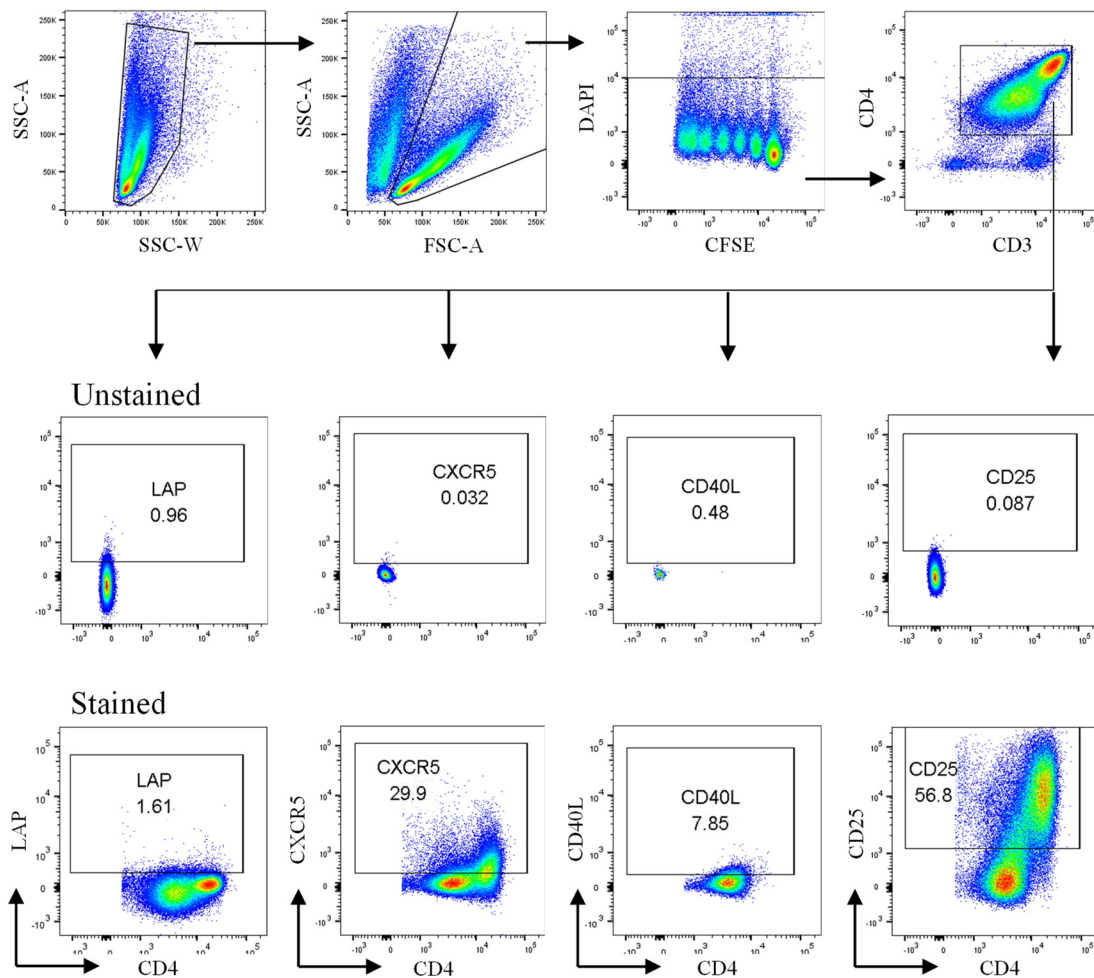


Figure 4. Gating strategy for activation analysis of naïve and memory CD4⁺ T-cells.

Naïve and memory CD4⁺ T-cells were stained with activation panel of antibodies prior to acquisition on the flow cytometer. Cells were gated on SSC-A v SSC-W to exclude debris followed by SSC-A v FSC-A to gate lymphocytes. Cells were further gated on DAPI⁻ cells and CD4⁺ T-cells were gated as CD3⁺CD4⁺. Gates were set based on the unstained populations and transferred to fully stained populations. In the case of CD40L an FMO control was used.

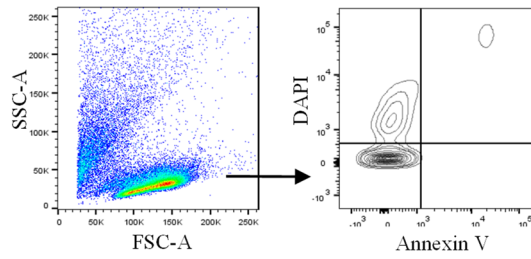


Figure 5. Gating strategy for viability of naïve and memory CD4⁺ T-cells. Naïve or memory CD4⁺ T-cells were stained with DAPI and Annexin V prior to acquisition on the flow cytometer. Cells were gated on SSC-A v FSC-A to gate lymphocytes. Viable cells were DAPI⁺ Annexin V⁻, gated based on clear positive and negative populations.

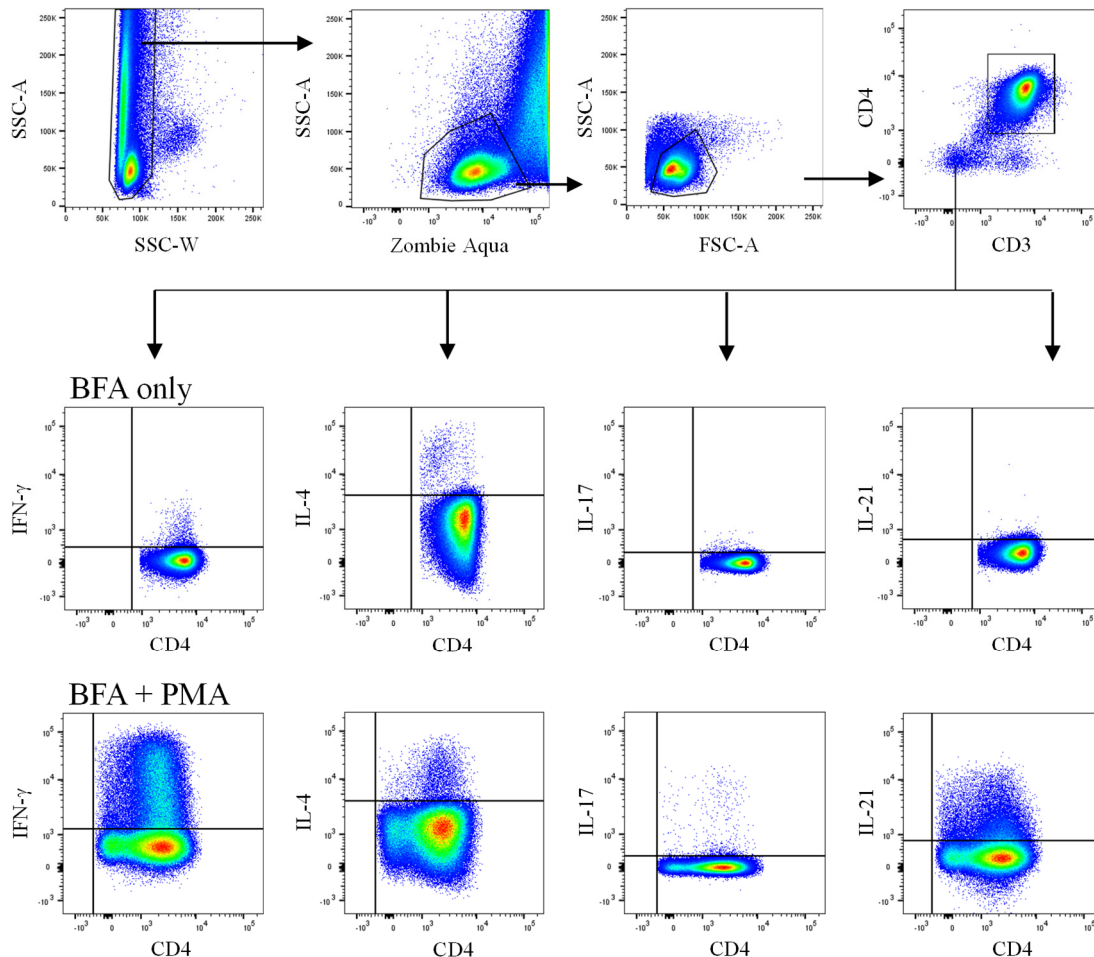


Figure 6. Gating strategy for intracellular cytokine staining of naïve and memory CD4⁺ T-cells. Naïve and memory CD4⁺ T-cells were stained with intracellular cytokine panel of antibodies prior to acquisition on the flow cytometer. Cells were gated on SSC-A v SSC-W to exclude debris followed by gating viable zombie aqua negative cells and SSC-A v FSC-A to gate lymphocytes. CD4⁺ T-cells were gated as CD3⁺CD4⁺. Gates were set based on the BFA only controls and transferred to naïve PMA/ionomycin stimulated populations.

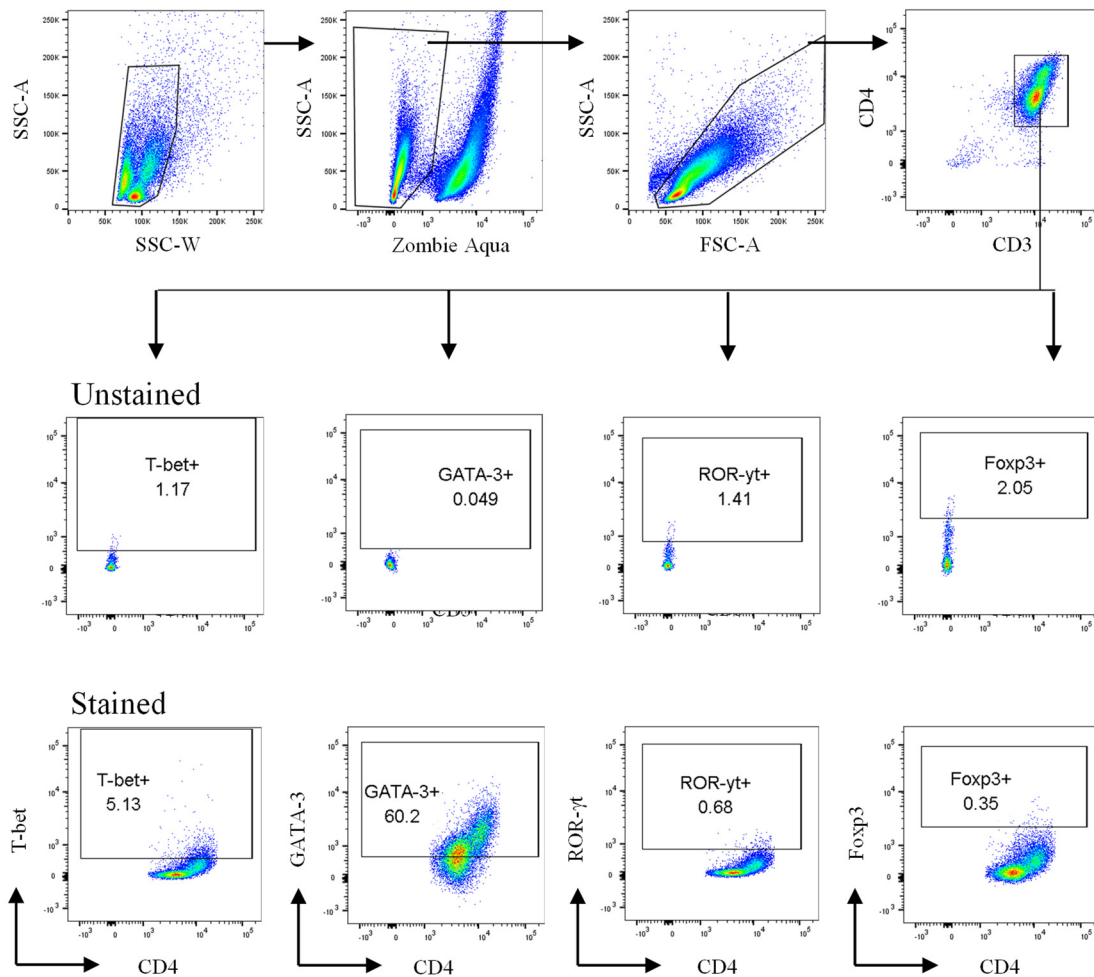


Figure 7. Gating strategy for transcription factor staining of naïve and memory CD4⁺ T-cells. Naïve and memory CD4⁺ T-cells were stained with transcription factor panel of antibodies prior to acquisition on the flow cytometer. Cells were gated on SSC-A v SSC-W to exclude debris followed by gating on viable zombie aqua negative cells and SSC-A v FSC-A to gate lymphocytes. CD4⁺ T-cells were gated as CD3⁺CD4⁺. Gates were set on an unstained population and transferred to fully stained naïve and memory populations.

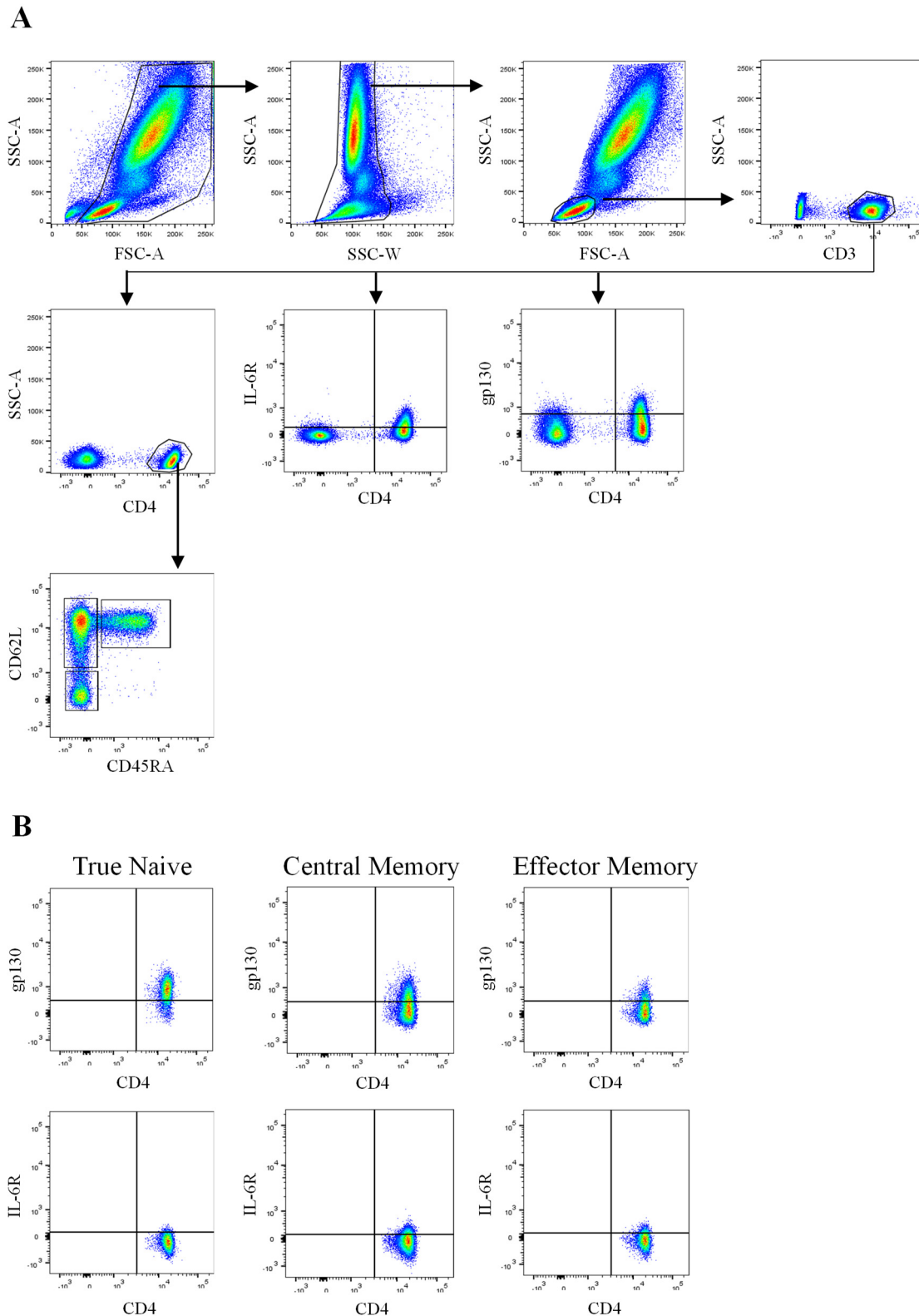


Figure 8. Gating strategy for whole blood phenotype staining of naïve and memory CD4⁺ T-cells. Whole blood was stained with a panel of antibodies prior to acquisition on the flow cytometer. Cells were gated on SSC-A v SSC-W to exclude debris followed by SSC-A v FSC-A to gate lymphocytes. CD4⁺ T-cells were gated as CD3⁺CD4⁺. True naïve CD4⁺ T-cells were identified as CD45RA⁺CD62L⁺, central memory CD4⁺ T-cells were identified as CD45RA⁻CD62L⁺ and effector memory CD4⁺ T-cells were identified as CD45RA⁻CD62L⁻. Gates were set based on the expression of IL-6R and gp130 on internal negative controls and transferred to naïve and memory populations.

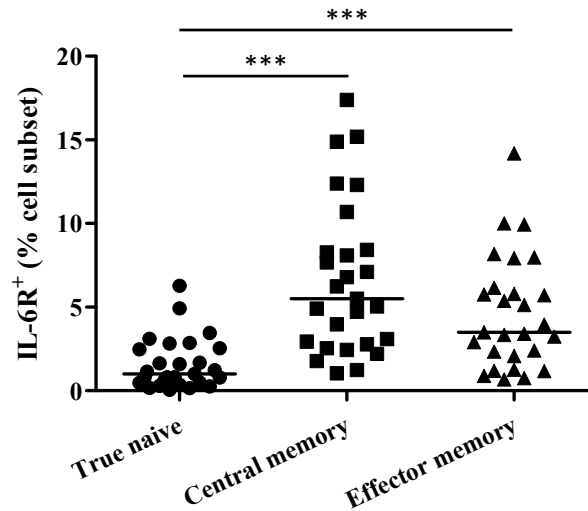


Figure 9. Expression of IL-6R on CD4⁺ T-cell subsets of early arthritis patients. Whole blood of early RA patients was stained for flow cytometry with a panel of antibodies to detect cell surface expression of IL-6R, using the IL-6R-CFS antibody recognising the 17506 clone. Cells were gated on SSC-A v SSC-W to exclude debris followed by SSC-A v FSC-A to gate lymphocytes. CD4⁺ T-cells were identified as CD3⁺CD4⁺ and from this population true naïve cells were identified as CD45RA⁺CD62L⁺, central memory cells identified by CD45RA⁻CD62L⁺ and effector memory cells identified as CD45RA⁻CD62L⁻. Horizontal bars represent the median value and significance was determined by non-parametric analysis of variance (Kruskal-Wallis) with Dunn's posthoc pairwise analyses: ***p<0.0001. N=27.

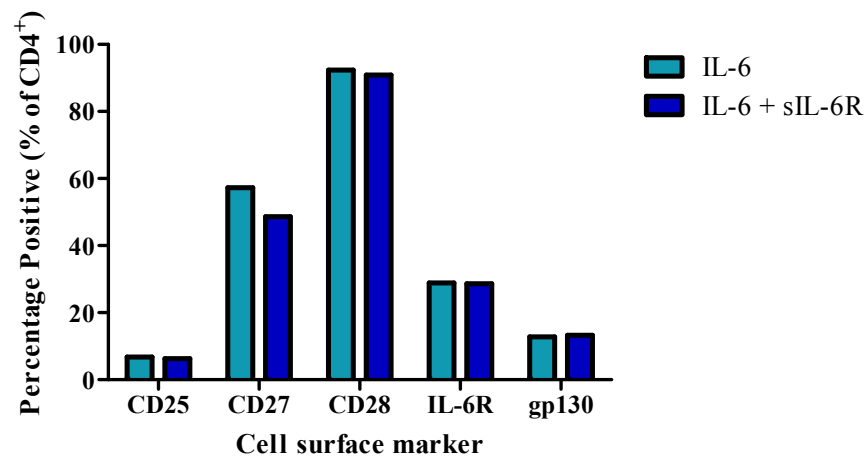


Figure 10. Surface marker expression of IL-6 treated and IL-6 with equimolar sIL-6R treated naïve CD4⁺ T-cells following 3 day culture. Freshly isolated naïve CD4⁺ T-cells were cultured for 3 days in TexMACS with 50ng/ml IL-6 or with 50ng/ml with equimolar sIL-6R. After 3 days cells were stained for cell surface markers for flow cytometry. Cells were gated as singlets, lymphocytes and then CD4⁺ T-cells were identified as CD3⁺CD4⁺, naïve CD4⁺ T-cells were identified as CD4⁺CD45RA⁺ and memory CD4⁺ T-cells identified as CD4⁺CD45RA⁻. N= 1.

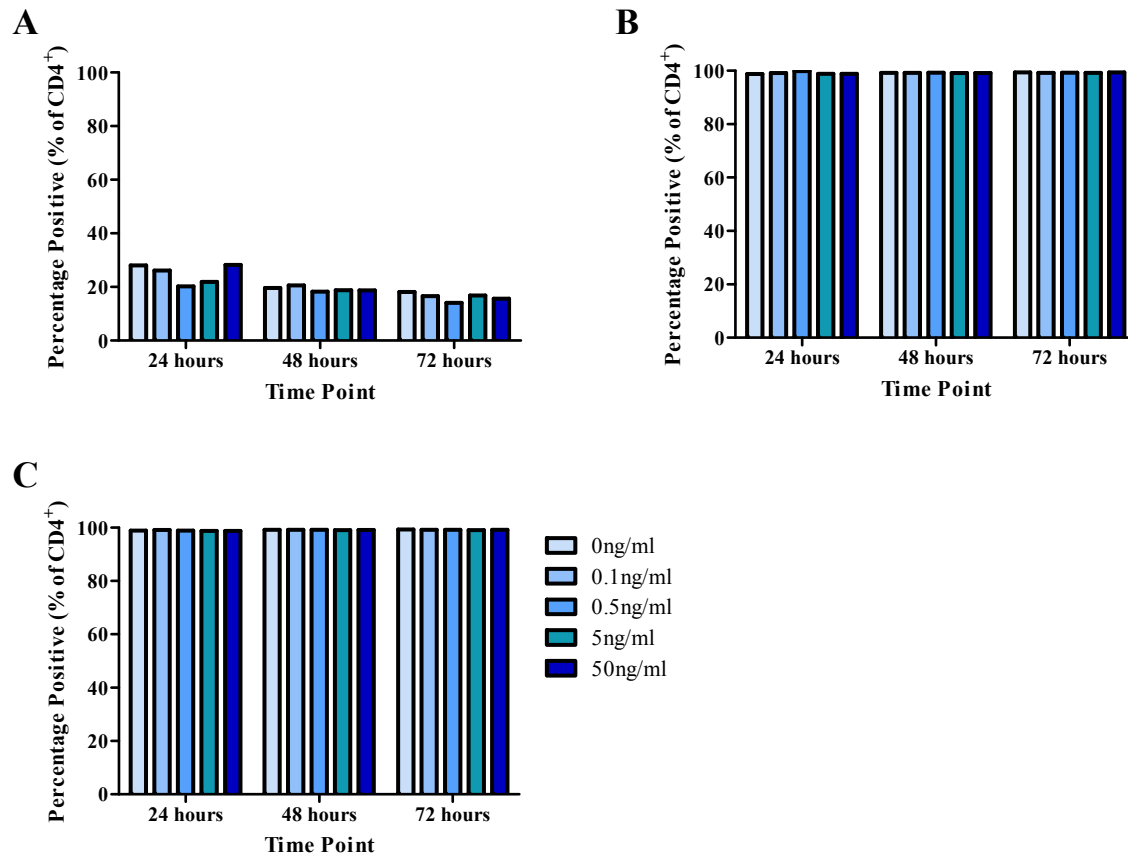


Figure 11. Expression of cell surface markers over 3 days culture with increasing concentrations of IL-6. Freshly isolated naïve CD4⁺ T-cells were cultured for 3 days in TexMACS alone, with 0.1ng/ml IL-6, 0.5ng/ml IL-6, 5ng/ml or 50ng/ml IL-6 all with equimolar sIL-6R. Cells were stained with phenotype panel of antibodies prior to acquisition on the flow cytometer. Cells were gated on SSC-A v SSC-W to exclude debris followed by SSC-A v FSC-A to gate lymphocytes. CD4⁺ T-cells were gated as CD3⁺CD4⁺. N=1

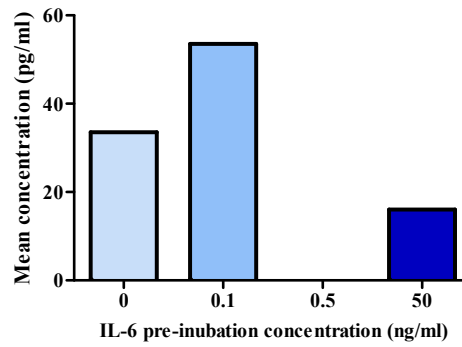


Figure 12. Cytokine production of naïve CD4⁺ T-cells after 3 days exposure to increasing concentrations of IL-6. Freshly isolated naïve CD4⁺ T-cells were cultured for 3 days in TexMACS alone, with 0.1ng/ml IL-6, 0.5ng/ml IL-6, 5ng/ml or 50ng/ml IL-6 all with equimolar sIL-6R. After 3 days supernatants were harvested and IFN, IL-10, IL-5 and IL-17 ELISA carried out. Results only obtained for IL-10. N=3.

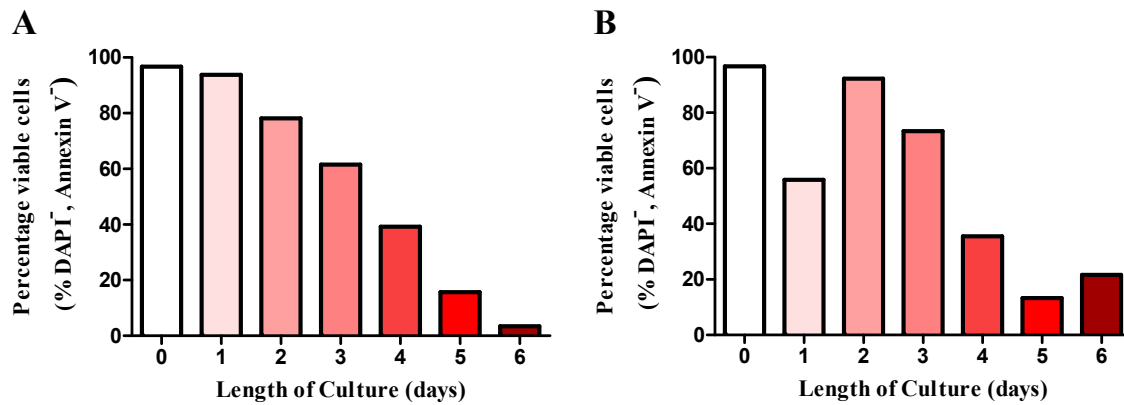


Figure 13. Percentage of viable cells over 6-day culture alone or with IL-6. Freshly isolated naïve CD4⁺ T-cells were cultured for 6 days in TexMACS alone or with 50ng/ml IL-6. Cells were stained with DAPI and Annexin V prior to acquisition on the flow cytometer. Cells were gated on SSC-A v FSC-A to gate lymphocytes. Viable cells were DAPI⁻Annexin V⁻.

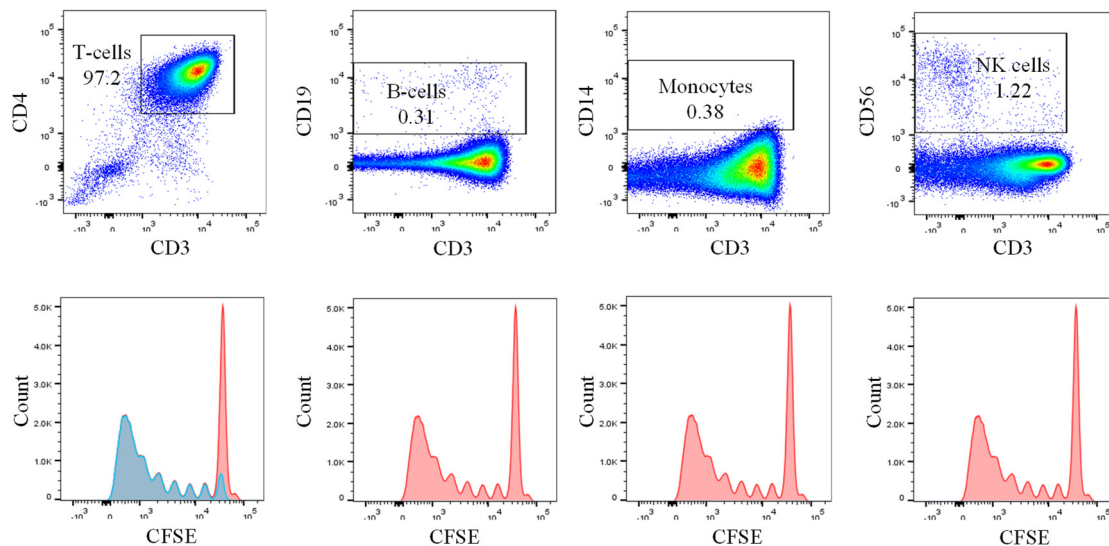


Figure 14. Examination of cell subsets in naïve CD4⁺ T-cell proliferation assay. Freshly isolated CFSE labelled naïve CD4⁺ T-cells were cultured for 3 days with, 0.5ng/ml IL-6 with equimolar sIL-6R. After exposure to IL-6 naïve CD4⁺ T-cells were washed and stimulated with 0.5µg/ml anti-CD3 and 1µg/ml anti-CD28 for a further 6 days. Following stimulation cells were stained with naïve purity panel of antibodies and acquired on the flow cytometer. Data analysed on FlowJo to give % of cells divided.

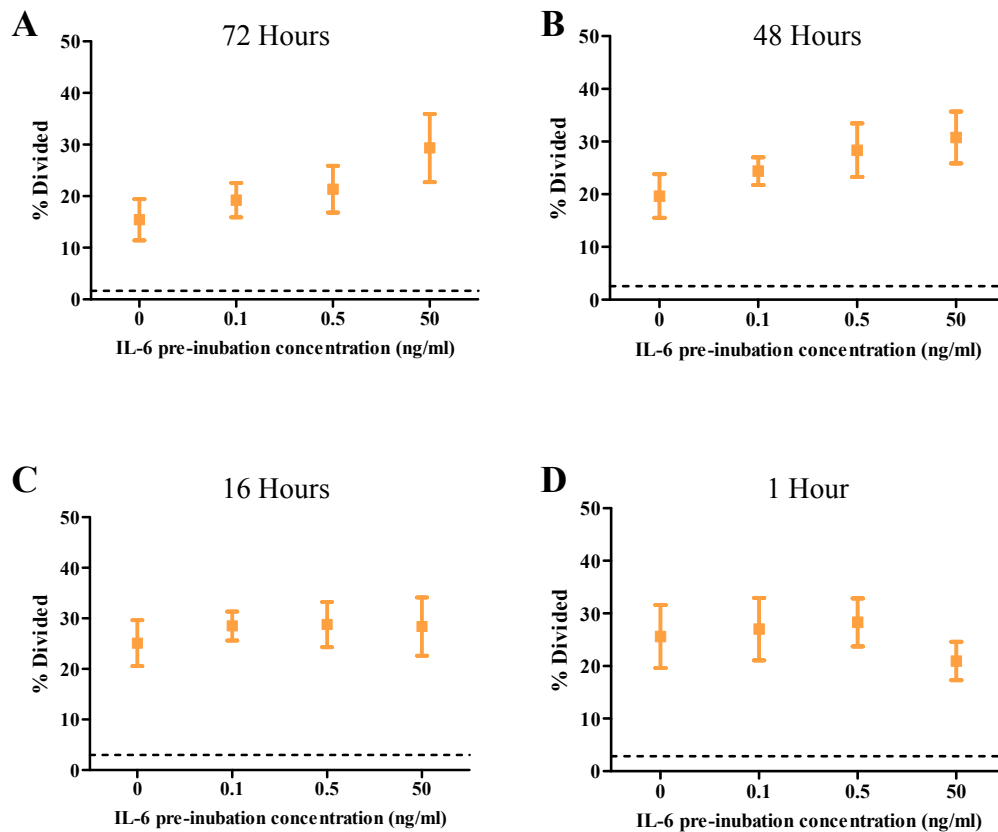


Figure 15. Proliferation of naive CD4⁺ T-cells following different lengths of pre-exposure to IL-6 and subsequent TCR stimulation. Freshly isolated CFSE memory naive CD4⁺ T-cells were cultured for 1 hour (A), 16 hours (B), 48 hours (C) or 72 hours (D) in TexMACS alone, with 0.1ng/ml IL-6, 0.5ng/ml IL-6 or 50ng/ml IL-6 all with equimolar sIL-6R. After exposure to IL-6 naïve CD4⁺ T-cells were washed and stimulated with a high stimulus; 1µg/ml anti-CD3 and 5µg/ml anti-CD28 or a low stimulus of 0.5µg/ml anti-CD3 and 1µg/ml anti-CD28 for a further 6 days. Following stimulation cells were acquired on the flow cytometer. Data analysed on FlowJo to give % of cells divided. Mean values were plotted with error bars representing SEM. Significance of each cell type and stimulus was determined by non-parametric analysis of variance (Friedman) with Dunn's posthoc pairwise analyses for individual IL-6 concentrations. N=3.

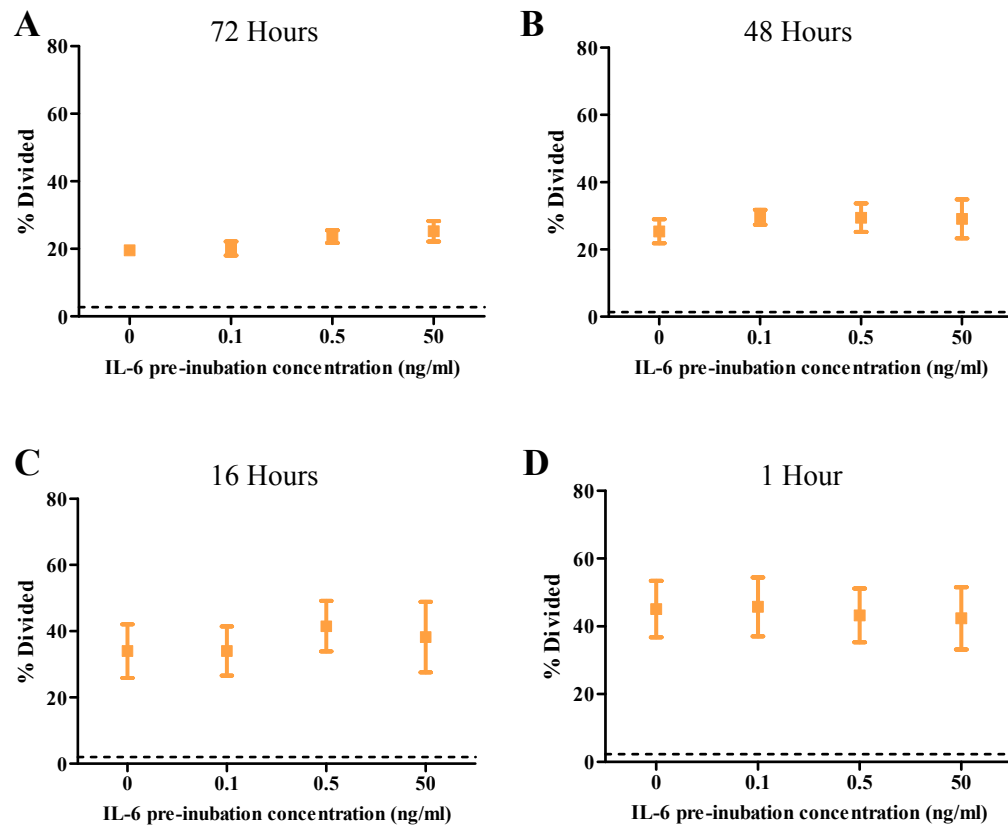


Figure 16. Proliferation of memory CD4⁺ T-cells following different lengths of pre-exposure to IL-6 and subsequent TCR stimulation. Freshly isolated CFSE memory naïve CD4⁺ T-cells were cultured for 1 hour (A), 16 hours (B), 48 hours (C) or 72 hours (D) in TexMACS alone, with 0.1ng/ml IL-6, 0.5ng/ml IL-6 or 50ng/ml IL-6 all with equimolar sIL-6R. After exposure to IL-6 naïve CD4⁺ T-cells were washed and stimulated with a high stimulus; 1µg/ml anti-CD3 and 5µg/ml anti-CD28 or a low stimulus of 0.5µg/ml anti-CD3 and 1µg/ml anti-CD28 for a further 6 days. Following stimulation cells were acquired on the flow cytometer. Data analysed on FlowJo to give % of cells divided. Mean values were plotted with error bars representing SEM. Significance of each cell type and stimulus was determined by non-parametric analysis of variance (Friedman) with Dunn's posthoc pairwise analyses for individual IL-6 concentrations. N=3.

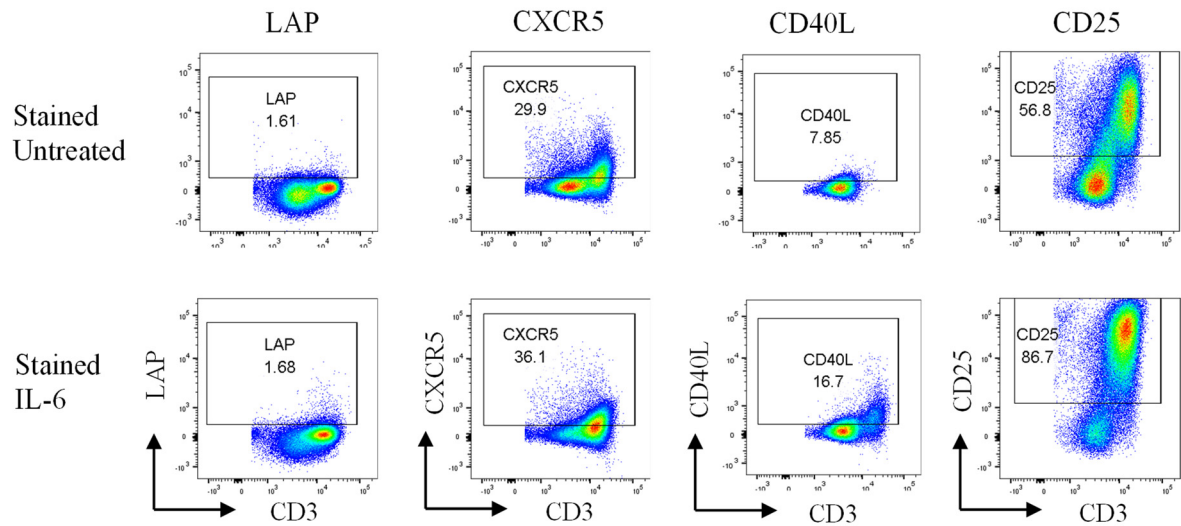


Figure 17. Activation marker expression of naive CD4⁺ T-cells following 3 days pre-exposure to IL-6 and subsequent 6 day TCR stimulation. Freshly isolated CFSE labelled naive CD4⁺ T-cells were cultured for 3 days in TexMACS alone or with 0.5ng/ml IL-6 with equimolar sIL-6R. Naïve CD4⁺ T-cells were washed and stimulated with a low stimulus of 0.5µg/ml anti-CD3 and 1µg/ml anti-CD28 for a further 6 days. Following stimulation cells were stained with activation marker panel of antibodies and acquired on the flow cytometer. Cells were gated on SSC-A v SSC-W to exclude debris followed by SSC-A v FSC-A to gate lymphocytes. CD4⁺ T-cells were gated as CD3⁺CD4⁺.

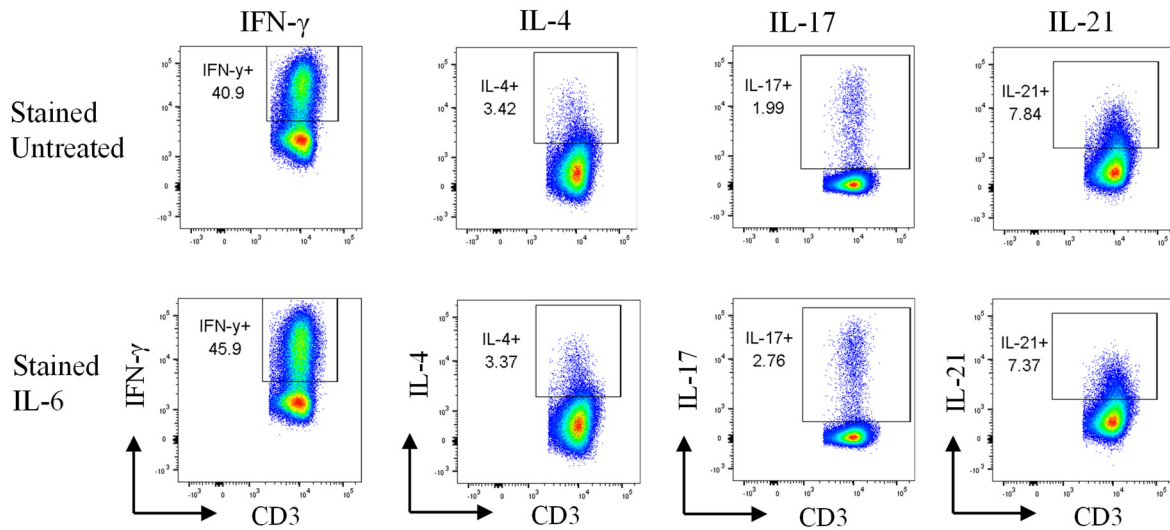


Figure 18. Cytokine production of naive CD4⁺ T-cells following 3 days pre-exposure to IL-6 and subsequent 6 day TCR stimulation. Freshly isolated CFSE naive CD4⁺ T-cells were cultured for 3 days in TexMACS alone or with 0.5ng/ml IL-6 with equimolar sIL-6R. After exposure to IL-6 naïve CD4⁺ T-cells were washed and stimulated with a low stimulus of 0.5 μ g/ml anti-CD3 and 1 μ g/ml anti-CD28 for a further 6 days. Following stimulation cells were stained with intracellular cytokine panel of antibodies and acquired on the flow cytometer. Cells were gated on SSC-A v SSC-W to exclude debris followed by SSC-A v FSC-A to gate lymphocytes. CD4⁺ T-cells were gated as CD3⁺CD4⁺.

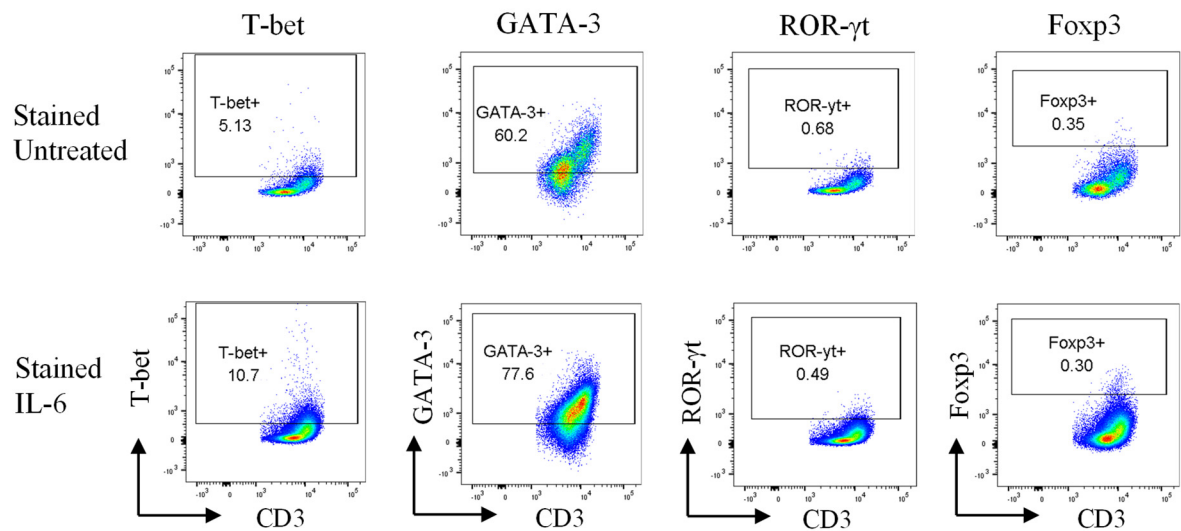


Figure 19. Transcription factor expression of naive CD4⁺ T-cells following 3 days pre-exposure to IL-6 and subsequent 6 day TCR stimulation. Freshly isolated CFSE naive CD4⁺ T-cells were cultured for 3 days in TexMACS alone or with 0.5ng/ml IL-6 with equimolar sIL-6R. After exposure to IL-6 naïve CD4⁺ T-cells were washed and stimulated with a low stimulus of 0.5μg/ml anti-CD3 and 1μg/ml anti-CD28 for a further 6 days. Following stimulation cells were stained with intracellular cytokine panel of antibodies and acquired on the flow cytometer. Cells were gated on SSC-A v SSC-W to exclude debris followed by SSC-A v FSC-A to gate lymphocytes. CD4⁺ T-cells were gated as CD3⁺CD4⁺.

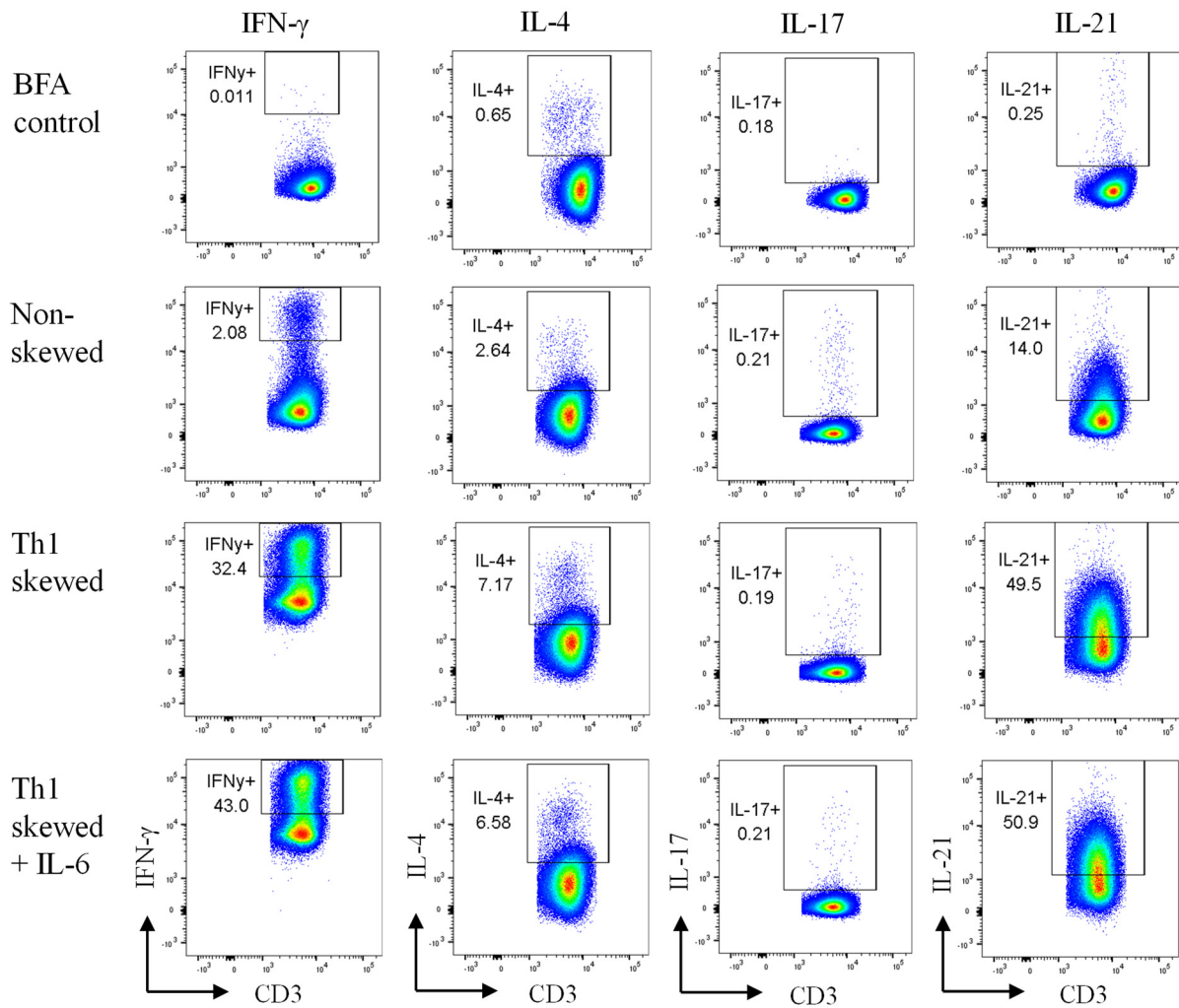


Figure 20. Cytokine production of CD4⁺ T-cells with or without differentiation towards Th1. Freshly isolated naïve CD4⁺ T-cells were cultured for 3 days in TexMACS. After 3 days cells were washed and differentiated towards Th1 in IMDM +10% FCS for 6 days. Following differentiation cells were rested for 1-4 days before stimulation with PMA/ionomycin and staining for intracellular cytokines for flow cytometry. Cells were gated as singlets, lymphocytes and then CD4⁺ T-cells were identified as CD3⁺CD4⁺. The top panel shows Th1 cells stimulated with BFA for controls, the second panel shows naïve CD4⁺ T-cells without skewing towards Th1, the third panel shows untreated Th1 cells and the bottom panel shows Th1 cells following IL-6 pre-exposure.

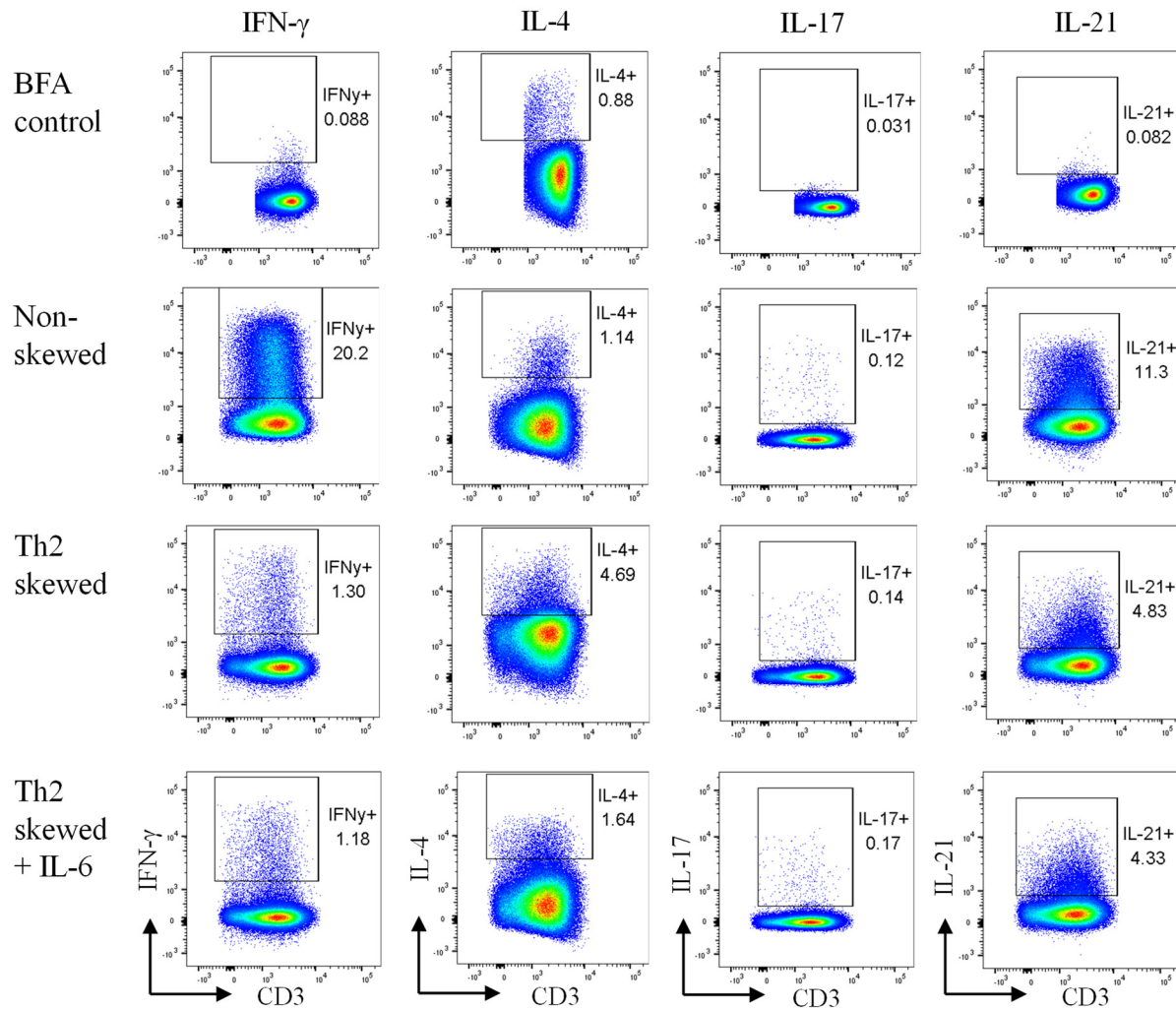


Figure 21. Cytokine production of CD4⁺ T-cells with or without differentiation towards Th2. Freshly isolated naïve CD4⁺ T-cells were cultured for 3 days in TexMACS. After 3 days cells were washed and differentiated towards Th2 in IMDM +10% FCS for 6 days. Following differentiation cells were rested for 1-4 days before stimulation with PMA/ionomycin and staining for intracellular cytokines for flow cytometry. Cells were gated as singlets, lymphocytes and then CD4⁺ T-cells were identified as CD3⁺CD4⁺. The top panel shows Th2 cells stimulated with BFA for controls, the second panel shows naïve CD4⁺ T-cells without skewing towards Th2, the third panel shows untreated Th2 cells and the bottom panel shows Th2 cells following IL-6 pre-exposure.

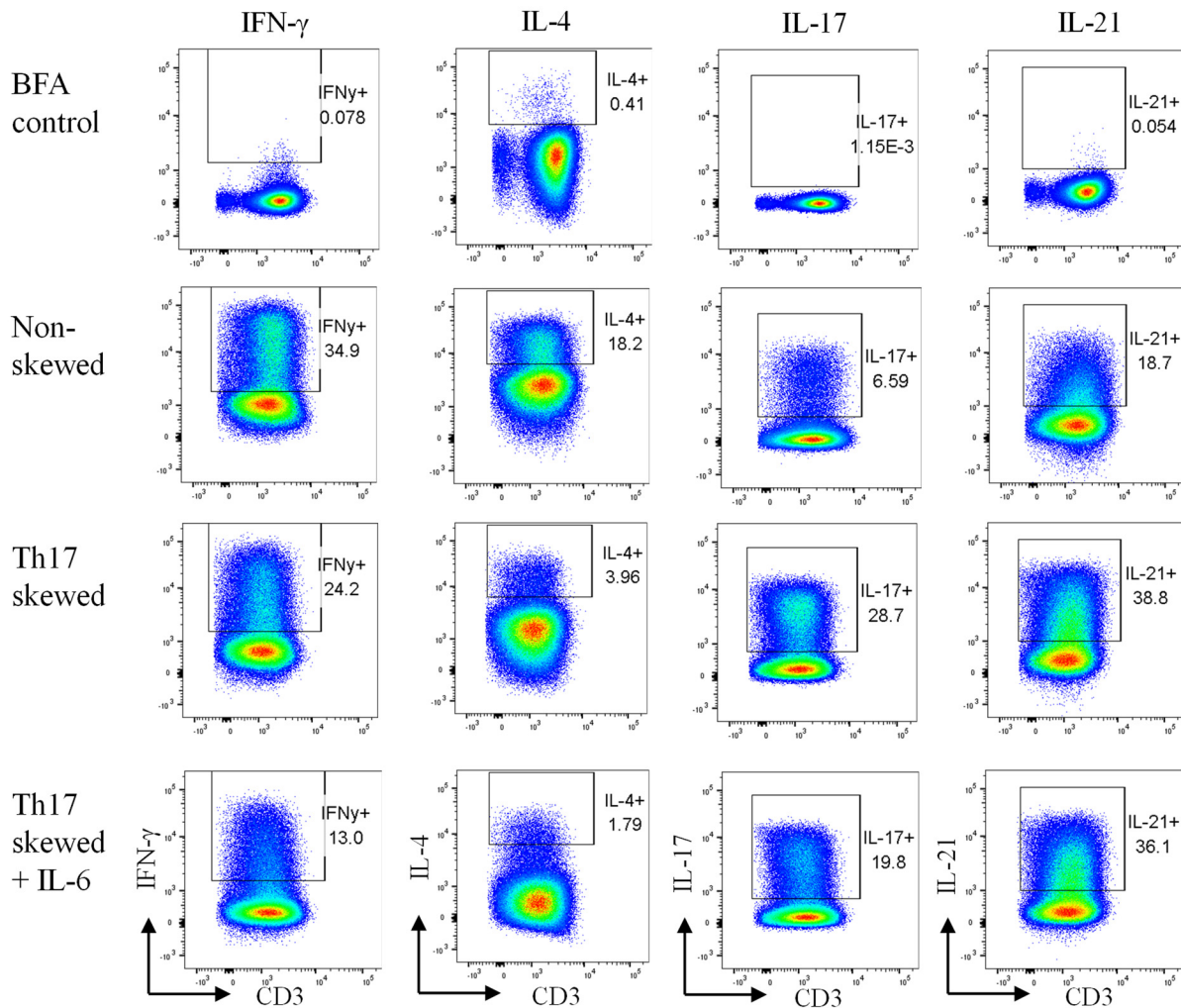


Figure 22. Cytokine production of CD4⁺ T-cells with or without differentiation towards Th17. Freshly isolated memory CD4⁺ T-cells were cultured for 3 days in TexMACS. After 3 days cells were washed and differentiated towards Th17 in IMDM +10% SR for 6 days. Following differentiation cells were rested for 1-4 days before stimulation with PMA/ionomycin and staining for intracellular cytokines for flow cytometry. Cells were gated as singlets, lymphocytes and then CD4⁺ T-cells were identified as CD3⁺CD4⁺. The top panel shows Th17 cells stimulated with BFA for controls, the second panel shows memory CD4⁺ T-cells without skewing towards Th17, the third panel shows untreated Th17 cells and the bottom panel shows Th17 cells following IL-6 pre-exposure.

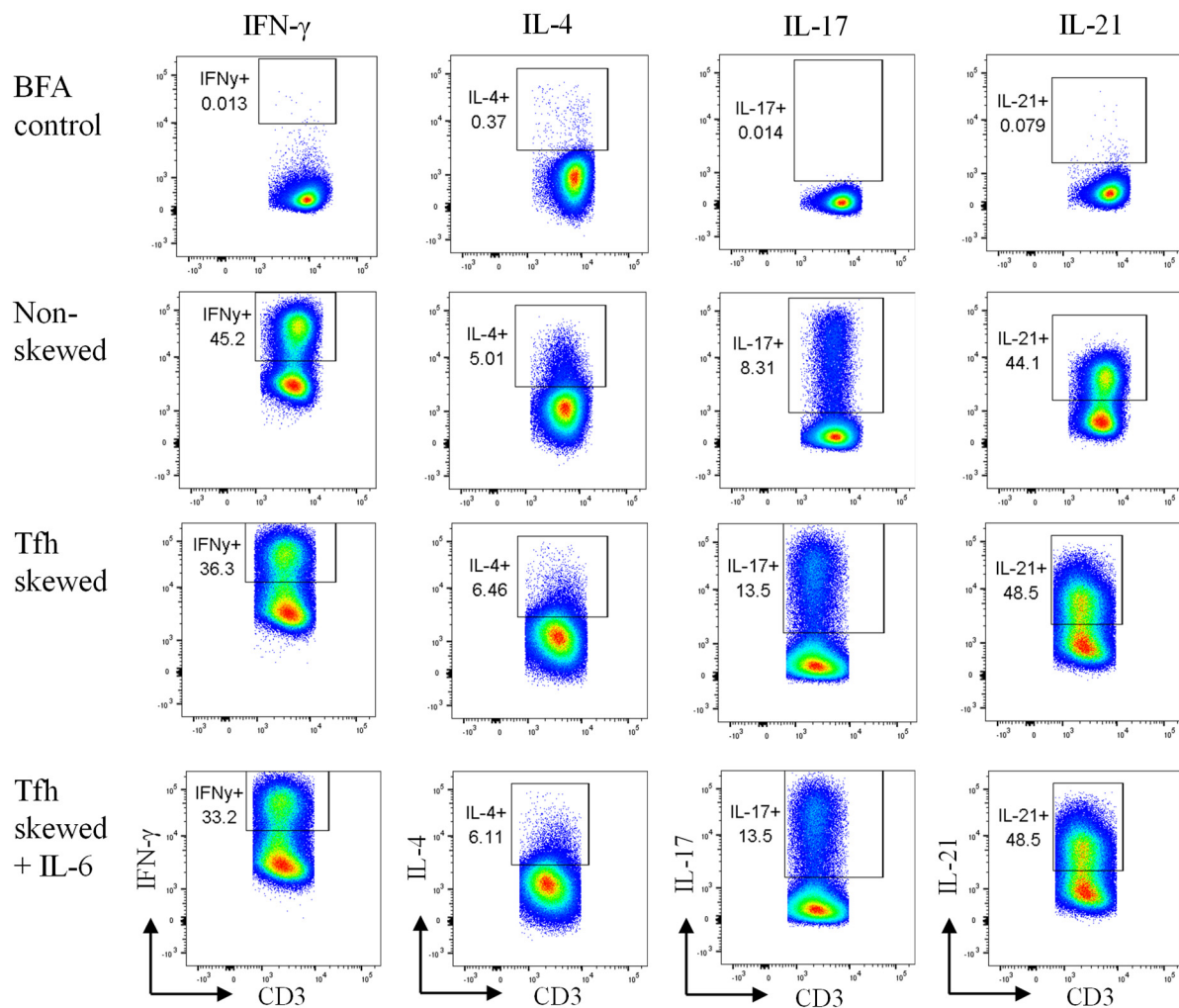


Figure 23. Cytokine production of CD4⁺ T-cells with or without differentiation towards Tfh. Freshly isolated CD4⁺ T-cells were cultured for 3 days in TexMACS. After 3 days cells were washed and differentiated towards Tfh for 6 days. Following differentiation cells were rested for 1-4 days before stimulation with PMA/ionomycin and staining for intracellular cytokines for flow cytometry. Cells were gated as singlets, lymphocytes and then CD4⁺ T-cells were identified as CD3⁺CD4⁺. The top panel shows Tfh cells stimulated with BFA for controls, the second panel shows CD4⁺ T-cells without skewing towards Tfh, the third panel shows untreated Tfh cells and the bottom panel shows Tfh cells following IL-6 pre-exposure.

Experiment	Condition	Total RNA	260:280
Figure Total CD4 cytokine stimulation Donor 1	1hr Untreated	95.5	1.97
	1hr IL-6	108	1.95
	1hr TNF	105.5	1.93
	1hr IFN	121	1.96
	1hr IL-21	109.7	1.93
	6hr Untreated	117.7	1.93
	6hr IL-6	115.3	1.96
	6hr TNF	114.2	1.99
	6hr IFN	111.2	1.99
	6hr IL-21	93.6	1.96
	24hr Untreated	102.3	2.01
	24hr IL-6	108.8	2.01
	24hr TNF	101	1.99
	24hr IFN	102.3	1.98
	24hr IL-21	98.7	1.98
Figure Total CD4 cytokine stimulation donor 2	1hr Untreated	2.9	1.95
	1hr IL-6	30.6	1.89
	1hr TNF	26.7	1.89
	1hr IL-21	27.2	1.83
	1hr IL-23	23.4	2.09
	1hr IL-27	21.8	2.07
	6hr Untreated	24.6	1.98
	6hr IL-6	20.5	2.08
	6hr TNF	26.6	1.99
	6hr IL-21	17.6	2.41
	6hr IL-23	15.8	2.13
	6hr IL-27	24.4	1.7
	24hr Untreated	31.7	1.79
	24hr IL-6	31.1	1.77
	24hr TNF	24.9	1.66
	24hr IL-21	29.7	1.84
	24hr IL-23	26.6	1.78
	24hr IL-27	35.7	1.86
Figure total CD4 cytokine stimulation donor 3	6hr Untreated	24.6	1.98
	6hr IL-6	20.5	2.08
	6hr TNF	26.6	1.99
	6hr IL-21	17.6	2.41
	6hr IL-23	15.8	2.13
	6hr IL-27	24.4	1.7
Figure total CD4 cytokine stimulation donor 4	1hr Untreated	25.2	1.99
	1hr IL-6	32.6	1.85

	1hr TNF	21.2	1.99
	1hr IL-21	38.6	1.82
	1hr IL-23	47.5	1.72
	1hr IL-27	40.9	1.74
	6hr Untreated	28.2	1.89
	6hr IL-6	28.9	2
	6hr TNF	26.7	2.02
	6hr IL-21	20.8	2.11
	6hr IL-23	39.5	1.7
	6hr IL-27	37.7	1.78

Table 1. Amount of RNA and 260:280 ratios from cytokine stimulated total CD4⁺ T-cells. CD4⁺ T-cells were stimulated with various pro-inflammatory cytokines to analyse STAT-3 target gene induction in *Figure 24*.

Experiment	Condition	Total RNA	260:280
Donor 1 Naïve CD4 ⁺ T-cells	Basal	57.3	1.99
	6hr Untreated	107.8	1.58
	6hr IL-6	65.4	1.8
	72hr Untreated	95.2	1.58
	72hr IL-6	78.9	1.75
	4hr Untreated	117.2	1.59
	4hr IL-6	91	1.76
	16hr Untreated	70.7	1.85
	16hr IL-6	80.5	1.78
	6 day Untreated	68	1.84
	6 day IL-6	92.1	1.96
Donor 1 Memory CD4 ⁺ T-cells	Basal	86.3	1.96
	6hr Untreated	106.9	1.81
	6hr IL-6	135.7	1.66
	72hr Untreated	124.8	1.63
	72hr IL-6	89.2	1.91
	4hr Untreated	104	1.68
	4hr IL-6	67.6	1.98
	16hr Untreated	109.5	1.67
	16hr IL-6	118.4	1.64
	6 day Untreated	159.2	2.02
	6 day IL-6	252.6	2.06
Donor 2 Naïve CD4 ⁺ T-cells	Basal	70.1	1.67
	6hr Untreated	43.3	1.91
	6hr IL-6	49.7	1.91

	72hr Untreated	42.5	1.91
	72hr IL-6	45.5	1.9
	4hr Untreated	126.8	1.52
	4hr IL-6	57.2	1.89
	16hr Untreated	98.2	1.58
	16hr IL-6	50.1	1.89
	6 day Untreated	89.1	1.94
	6 day IL-6	130.9	1.89
Donor 2 Memory CD4 ⁺ T-cells	Basal	55.2	1.92
	6hr Untreated	70.4	1.91
	6hr IL-6	81.1	1.78
	72hr Untreated	71.6	1.69
	72hr IL-6	53	1.93
	4hr Untreated	60	2.19
	4hr IL-6	77.8	1.83
	16hr Untreated	87.2	1.87
	16hr IL-6	89.7	1.74
	6 day Untreated	316.8	2.02
	6 day IL-6	394.8	2.01
Donor 3 Naïve CD4 ⁺ T-cells	Basal	85.8	1.78
	6hr Untreated	71.5	1.82
	6hr IL-6	64.9	1.99
	72hr Untreated	47.8	2.09
	72hr IL-6	128.2	1.61
	4hr Untreated	71	1.88
	4hr IL-6	59.6	1.95
	16hr Untreated	98.6	1.62
	16hr IL-6	65	1.84
	6 day Untreated	196.7	1.85
	6 day IL-6	431.7	1.98
Donor 3 Memory CD4 ⁺ T-cells	Basal	13.1	1.82
	6hr Untreated	23.6	1.53
	6hr IL-6	55.6	1.51
	72hr Untreated	22	1.54
	72hr IL-6	33.1	1.47
	4hr Untreated	47.9	1.48
	4hr IL-6	34.1	1.67
	16hr Untreated	59.8	1.54
	16hr IL-6	28.8	1.65
	6 day Untreated	18.6	2.02
	6 day IL-6	61.8	1.98
Donor 4 Naive CD4 ⁺ T-cells	Basal	54.4	1.71
	6hr Untreated	35.3	1.81
	6hr IL-6	93.1	1.55
	72hr Untreated	90.2	1.54
	72hr IL-6	41.2	1.67

	4hr Untreated	42.8	1.64
	4hr IL-6	70.8	1.57
	16hr Untreated	96.2	1.54
	16hr IL-6	48.2	1.72
	6 day Untreated	65.5	1.43
	6 day IL-6	26.2	1.49
Donor 4 Memory CD4 ⁺ T-cells	Basal	114.4	1.72
	6hr Untreated	94.5	1.85
	6hr IL-6	113.9	1.79
	72hr Untreated	93.7	1.76
	72hr IL-6	80.2	1.89
	4hr Untreated	99.5	1.77
	4hr IL-6	68.7	1.73
	16hr Untreated	127.1	1.7
	16hr IL-6	72.3	1.69
	6 day Untreated	87.7	1.82
	6 day IL-6	31.1	1.69

Table 2. Amount of RNA and 260:280 ratios from naïve and memory CD4⁺ T-cells samples for microarray. Naïve and memory CD4⁺ T-cells were isolated from healthy donors prior to culture as per the in vitro model with RNA taken at multiple experimental time-points.

Healthy donors for microarray	Age	Sex
Donor 1	40	Male
Donor 2	25	Male
Donor 3	24	Male
Donor 4	37	Female
Donor 5	38	Female

Table 3. Characteristics of healthy donor samples used for microarray. Age and sex of healthy donors whose blood was taken for use in microarray.

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